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14. ABSTRACT

Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss in military personnel due to non-penetrating traumatic injuries to the eyes and brain visual centers, likely caused by blast shock waves. In light of the difficult lifelong disability that permanent loss of vision represents, we propose there is an urgent need for new drug therapies that can arrest progression of neuronal cell death in the eye (retina) and brain, as result of exposure to blast waves. Our hypothesis is that novel omega-6 and omega-3 polyunsaturated fatty acid derived lipid mediators of inflammation, i.e., lipoxins, neuroprotectins, and resolvins, will aid as drugs in healing of neurons critical to visual function after blast wave induced eye and brain injuries. Using an adult rat model of blast wave exposure, during the first phase of the study, we rigorously characterized the cellular and functional damage to the eyes (retinas) and brain visual centers, by electroretinography (ERG), visual discrimination behavioral testing, and histopathology. Blast wave injury was carried out by placing the rats in a compressed air driven shock tube and exposing them, in a right side on orientation, once to a 20 psi (260 Hz) blast over pressure wave. Rats were assessed at baseline and then 1, 7, and 14 days post-exposure. By 7 to 14 days out, blasted rats versus shams showed significantly decreased ERG waveform amplitudes of retinal response to a light flash stimulus for the right side eyes (~ 30% less; n = 15 vs.14), a trend for impaired ability to visually discern a cue light to earn food rewards (~ 30% less; n = 10 vs. 11), and significant neuronal cell degeneration within the right side retinas and both brain optic tracts (2 and 3-fold more, respectively; n = 15 vs. 14). ERG and histopathology results significantly correlated with each other (r = - 0.7). There also was a strong relationship between the retina and brain optic tract cell damage (r = 0.8). Overall, our findings demonstrate that blast wave exposure leads to loss of vision in rats, likely through retinal cell death followed by anterograde degeneration of brain visual centers from lack of signaling input.

During the second phase of the study, we examined the efficacy of four drugs known to be polyunsaturated fatty acid derived lipid mediators of inflammation, i.e., lipoxin A4 (LXA4), protectin DX (PDX), resolvin D1 (RVD1), and resolvin E1 (RVE1), to alleviate the blast-induced neuronal cell damage to the retinas and brain. Rats were exposed to a single blast overpressure wave, as previously described. The drugs were immediately administered (< 15 min) to the blasted rats by intravenous injection (25 µg/kg), and then given every other day out to 14 days. As before, neuronal health status was evaluated by ERG, visual discrimination behavior, and histopathology (n = 12, 11, 12, and 12, respectively), at baseline and then out to 14 days post-injury. ERG of their right eyes - that faced the blast - showed an apparent drug efficacy order of LXA4 > RVE1 ≈ PDX ≈ RVD1. Visual discrimination testing of both eyes combined, yielded an efficacy order of PDX > RVE1 > LXA4 ≈ RVD1. Histopathology of their right retinas and innervated left brain optic tracts gave an efficacy order of LXA4 ≈ RVE1 ≈ PDX > RVD1 and LXA4 > RVD1 > PDX ≈ RVE1, respectively. Overall, LXA4 had the greatest positive effect. For all outcome measures, however, the four drugs produced modest, if any, injury-improvements for the blasted retina and brain, suggesting they have a rather limited potential as visual system therapeutics. Failure of this treatment approach, however, maybe due to ineffective delivery of the drugs to the neuronal injury sites as a result of systemic dilution, transient in *vivo* half-lives, and/or poor passage across the blood-brain / retinal barriers.

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INTRODUCTION

Blast injury has emerged as arguably the greatest threat to warfighters in current Mideast theaters of operation (Warden, 2006), and is the leading cause of vision loss in military personnel (Cockerham, 2011; Capó-Aponte, 2012). Of blast-related casualties, 43% display closed eye injuries having a 26% incidence of retina damage (e.g., hemorrhaging, tears, and detachments), which is very consistent with a blast wave displacement of fragile ocular tissues (Cockerham, 2011). Veterans with blast related neurotrauma often report chronic symptoms of photophobia and visual tracking / field impairments, e.g., saccades, abnormal accommodation, acuity / contrast sensitivity decreases, and quadrantanopia (Goodrich, 2013; Lemke, 2013). Although soldiers are issued protective goggles in the field, ocular injuries can still result due to non-compliance of wear, blast wave penetration, or being blown off the face (Lemke, 2013). It is also possible that the brain visual centers are being directly affected, since it is well established that blast wave exposure causes traumatic brain injuries (Warden, 2006). Despite the difficult lifelong disability that permanent loss of vision represents, there are currently only a modest number of studies in animals that have attempted to assess blast wave injuries to the visual system (Petras, 1997; Koliatsos, 2011; Hines-Beard, 2012; Jiang, 2013; Mohan, 2013; Zou, 2013; Bricker-Anthony, 2014a, b; Dutca, 2014; Wang, 2014; Bricker-Anthony, 2015; Choi, 2015). Many of these prior studies fall short on the soundness of experimental design (e.g., poor blast simulation and/or non-inclusive outcome measures); and only two have looked at potential drug treatments using agonists to the β-adrenergic receptor and nicotinamide phosphoribosyl transferase, as delivered by topical application to the cornea or systemically by intraperitoneal injection, respectively (Jiang, 2013; Dutca, 2014). First, we purposed to rigorously characterize the cellular, neuronal signaling, behavioral pathology of blast wave injuries to the eyes, specifically the retinas, and brain visual centers of adult male rats. These eye injury studies were more aptly carried out, in accordance with others, by subjecting the animals to high fidelity simulated blast over pressure waves (Friedlander waveform), as produced by a compressed air driven shock tube (Petras, 1997; Koliatsos, 2011; Wang, 2014; Choi, 2015). Eye and brain injuries are assessed by us in the rats out to 14 days post-exposure, using well established techniques of electroretinography (ERG; retinal signaling response with a light stimulus), visual discrimination behavioral testing (pressing a lever with a variable cue light to earn a food reward), and histopathology (H&E and silver stains for neuronal cell degeneration). Second, we purposed to develop new drug therapies that can arrest progression of neurodegeneration in the retina and brain, as result of exposure to blast waves. Our hypothesis is that novel polyunsaturated fatty acid derived lipid mediators of inflammation, i.e., lipoxins, neuroprotectins, and resolvins, will aid as drugs in healing of neurons critical to visual function after damage from blast wave exposure. Structurally, these lipids are stereo-specific hydroxylated derivatives of the omega-6 and omega-3 fatty acids, arachidonic (20:4ω-6), eicospentaenoic (20:5ω-3), and docosahexaenoic (22:6ω-3) acids (see supplemental Figure B, below). Indeed, all of these endogenously produced molecules have been shown to heal ischemic, mechanical, and disease injuries to the retina and brain (Serhan, 2008; Bazan, 2010; Serhan, 2010). Targets for these molecules are G-protein coupled immune-factor receptors on the surfaces of white blood cells (Serhan, 2011). Their basic mode of action is to stop neutrophil migration; block cytokine and eicosanoid release; and recruit monocytes for apoptotic cell removal; and thus, they promote wound healing by moving an acute injury state toward a resolution phase, as opposed to entering a chronic state leading to cellular apoptosis and eventual tissue fibrosis (Serhan, 2010) (see supplemental figure C, below). Thus, we felt that they were excellent drug candidates for our neuronal injury model; and we screened four - commercially available - sound examples, i.e., lipoxin A4, protectin DX, resolvin D1, and resolvin E1. Each drug was intravenously administered by tail vein injection to the rats immediately following blast exposure and then every other day out to 14 days thereafter. Assessment of drug efficacy at alleviating retina and brain neuronal cell damage was carried out using the previously described outcome measures. Overall, results from our study will provide an important contribution to the understanding and therapy of blast related injuries as translated to man, and thus to the advancement of military as well as civilian medicine.

KEYWORDS

Rat, brain, eye, retina, blast wave, inflammation, neuronal cell degeneration, drug therapeutics, lipid mediatiors, lipoxin A4, protectin DX, resolvin D1, resolvin E1, electroretinography, ERG, visual discrimination, and histopathology.

OVERALL PROJECT SUMMARY

I. Induction of Eye and Brain Injuries using Exposure to Blast Overpressure Waves

Materials and Methods:

Adult male Sprague Dawley rats (2 months-old) are placed under brief anesthesia using isoflurane gas. Anesthetized animals are put in a prone transverse position inside a nylon mesh sling that is secured to a metal frame sled. Rats are positioned with right side of the body perpendicular and opposite to the sled, and hence right eye facing the oncoming blast wave during exposure. In this manner, the left eye serves as a control, expected to incur less severe injuries or none. The rat-loaded sled is inserted down the barrel of a compressed air driven shock tube to a preset position (~ 2 ft.) in its forward expansion chamber. The unawake animal is then exposed to a single air driven blast wave with a main harmonic frequency at 260 Hz and a peak over pressure of 138 kPa (20 psi). The blast wave is generated and propagated down the shock tube by a rapid-buildup compressed air rupturing of a Mylar membrane, of predetermined thickness, to deliver 20 psi of air to the rat's position, as clamped between the rear compression and forward expansion chambers. The blast wave travels by the rat with a Mach 1.34 shock front speed, 62 μsec rise time, 6 msec duration, 281 mph (126 m/s) wind speed, and an acceleration g-force of > 1000 g. Blasted rats are immediately removed from the shock tube and monitored on a thermal blanket during recovery. Animals exhibiting stable respiration and awakening signs are returned to their home cages. If signs of respiratory failure (apena) are noted, then cardiopulmonary resuscitation (CPR) is performed by blowing oxygen into the lungs and massaging the chest. Typically, CPR is able to restore the rat's breathing reflex within several minutes, reducing the risk of retina and brain damage from prolonged hypoxia. Shams are subjected to isoflurane anesthesia and recovery steps as described above, but not blast waves. Blasted rats are also subjected to treatment after injury with experimental drugs by their administration immediately post-blast using intravenous injection, as to be described below (section II). Sham and blasted rats are then used for ERG or visual discrimination behavioral testing, as to be detailed later (sections III and IV).

Results and Specific Conclusions:

Over the entire study, we successfully exposed a total of 84 rats to blast waves along with 24 aged matched shams, which underwent all outcomes measures (see supplemental Figure A). This blast wave exposure procedure has been well established in our laboratory for producing mild to moderate traumatic brain injuries in rats, usually with accompanying retina damage. Figure 1, below, shows a diagram of the compressed air driven shock tube for exposing the rats to blast over pressure waves, as detailed above. As shown in Figure 2, in eyes collected at 14 days post-blast, the exposure leads to obvious exterior damage that is still present as distinct bruising on the lower portion of the sclera (i.e., contusions), as well as corneal base redness and cloudiness of the lens. We have also noted shortly after the blast the presence of hemorrhaging down inside the vitreous humor, as visible through the pupil. Scoring of the external eye injuries on a rank scale of 1 - 6 (i.e., none, minimal, mild, moderate, severe, and catastrophic) showed that the right eye facing the blast was significantly damaged versus shams (2-fold; n = 14 and 15, respectively), with an injury incidence of 67%. The left eye, however, also frequently displayed injuries (47% incidence), likely due to propagation of the blast wave through the skull, wrapping around the head, and/or reflections off of the shock tube walls. One major concern that we had with this technique is the potential for Mylar membrane fragments or animal holder netting to strike the rat's eyes and cause extraneous injuries during the blast wave generation. Indeed, some of the contusion marks

we observed on the rat's eyes post-blast were high up on the sclera near the corneal base, suggestive of netting or Mylar fragment strikes. Consistent with this, we often found Mylar dust at the position of the rat in the expansion chamber following blast; and occasionally microscope fragments of Mylar were embedded in the animal's cornea. We considered putting protective gauze patches over the rat's eyes, but this could lead to dampening or distortion of the blast wave upon impact. Another major concern we had with the procedure is many rats come out of the shock tube exhibiting severe signs of apnea. If breathing ceased, we immediately perform CRP (chest massage and oxygenation) on the animal until vital signs were restored. We only had a few rats die during blasting or within 24 hours afterwards, yielding an excellent survival rate. Overall, the blast mortality incidence was 2%, which is considerably lower than a 20% death rate that we had originally predicted. Also, only a couple rats fully lost an eye from the blast exposure, which represents an extremely low incidence in our model of non-treatable blindness due to the injury (< 3%). This procedure, however, could still have produced transient ischemia in rats afflicted with apnea. It is known that the retina and brain are hypersensitive to lack of oxygen; and thus ischemic conditions could exacerbate any neuronal cell damage due to blast alone. If respiratory failure impacts are a major concern, intubation and mechanical ventilation of all rats for a short time period immediately post-blast could be considered.

Figure 1: Diagram of WRAIR shock tube for generating blast waves

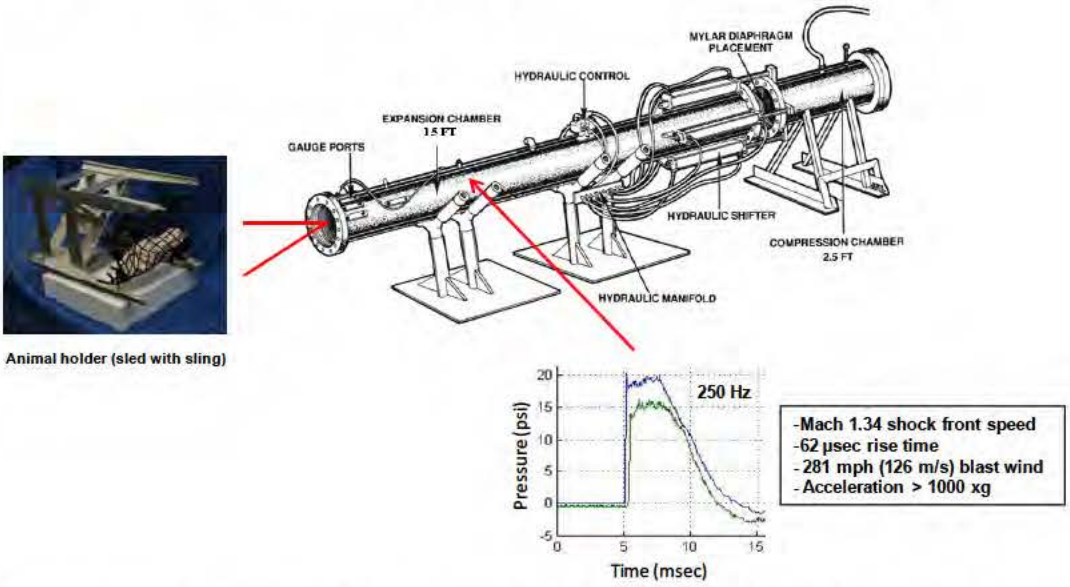


Figure 1. Representative diagram of a compressed air driven shock tube used to generate blast over pressure waves for inducing neuronal injuries to the eyes (retina) and brain visual centers of rats. The rat-loaded sled (inset) is inserted down the barrel of the shock tube to a preset position (~ 2 ft.) in its forward expansion chamber. The unawake animal is then exposed to a single air driven blast wave with a main harmonic frequency at 260 Hz and a peak over pressure of 138 kPa (20 psi). The blast wave is generated and propagated down the shock tube by a rapid-buildup compressed air rupturing of a Mylar membrane, of predetermined thickness, to deliver 20 psi of air to the rat's position, as clamped between the rear compression and forward expansion chambers. The blast wave travels by the rat with a Mach 1.34 shock front speed, 62 µsec rise time, 6 msec duration, 281 mph (126 m/s) wind speed, and an acceleration g-force of > 1000 g.

Figure 2: Eye globe injuries and relative damage scores of sham and blasted rats.

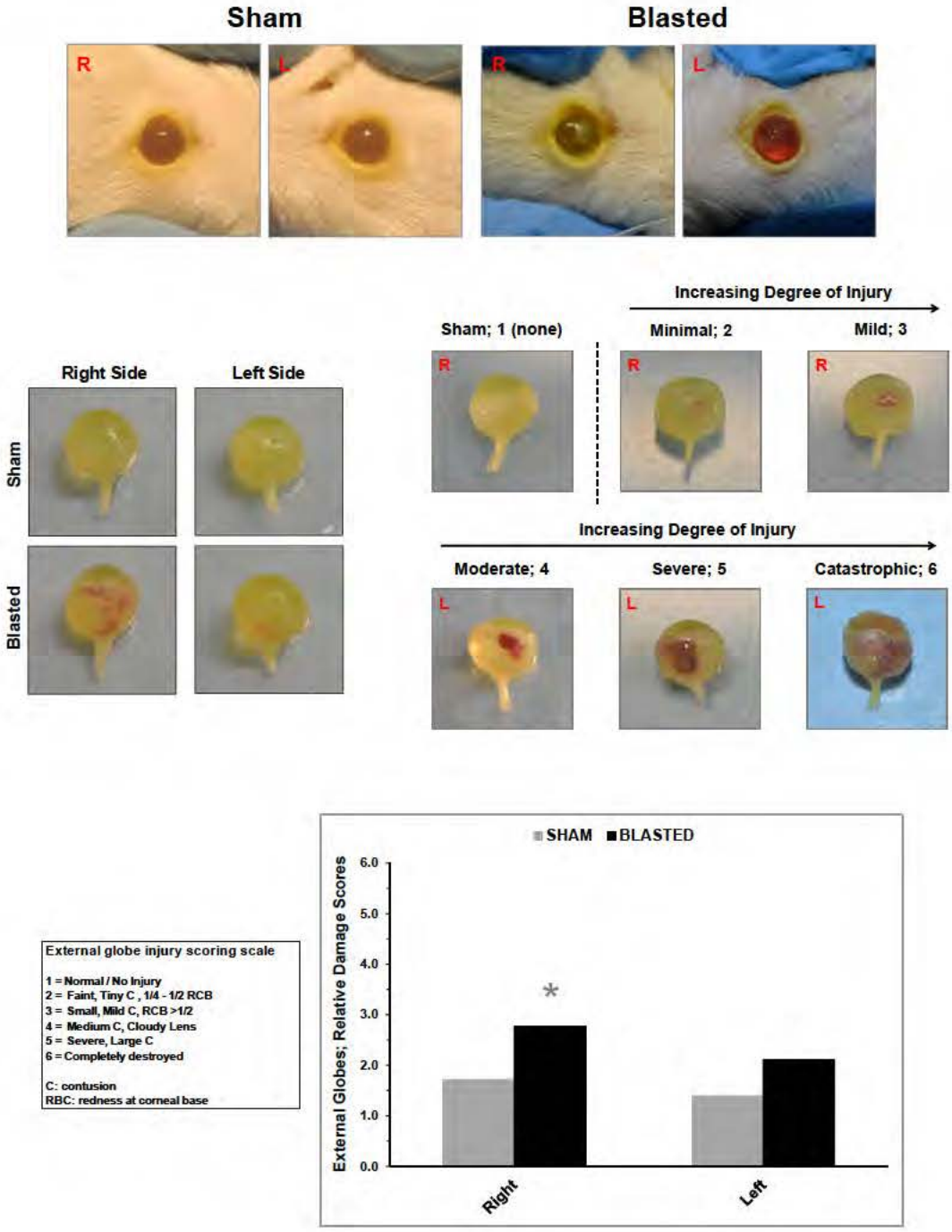


Figure 2. Top panels; representative eye globes (right and left) from sham and blasted rats collected at 14 days post-exposure. Blasted eye sclera show distinct red contusion marks. Bottom panel; bar graphs for relative damage scores of external globe injuries (right and left) of sham and blasted rats (means only; n = 14 and 15, respectively). Rank scale (1 - 6) used for scoring is shown in detail in the left inset. *$p \leq 0.05$ vs. shams, as carried out by Mann-Whitney U test for non-parametric data. Likewise, standard deviations are not given, as is appropriate for this type of data.

II. Administration of Experimental Drugs to Blasted Rats.

Materials and Methods:

During the study, we tested the efficacy of four novel anti-inflammatory drugs in the rats following blast wave exposure, i.e., lipoxin A4, protectin DX, resolvin D1, and resolvin E1 (n = 12, 11, 12, and 12, respectively). These compounds are endogenously occurring metabolites (hydroxylated derivatives) of omega-3 and omega-6 polyunsaturated fatty acids, known to be potent pro-resolving mediators of inflammation (see supplemental Figure B). They act by binding to immune receptors on white blood cells and inhibit activities that exacerbate tissue necrosis (e.g., cytokine release) (see supplemental Figure C). The drugs were all purchased from the Cayman Chemical Company (Ann Arbor, MI) as stocks in absolute ethanol, and were routinely stored at -80^{o}C. Unlike the other compounds that are kept in supply at Cayman Chemicals, resolvin E1 was custom synthesized for us as an exclusive product. In the course of the experiments, two batches of each drug were ordered, due to the need to replace the original stocks that were severely compromised and thus disposed of after a storage freezer failure late in the study. We had already utilized the original stocks for experiments on ~70% of the drug treated animals (i.e., 2 shams and 5 - 6 blasted rats each). Quality control (QC) assurance data was requested from Cayman for each batch / stock, as determined by liquid chromatography / mass spectrometry. Shown below in Table 1 are the QC results (purity, molecular mass, and concentration) from the issued certificates of analysis. These values confirm that as shipped to us all of the drugs stocks were of extremely high purity and structural integrity, and at a reasonable concentration for taking what is needed by volume.

Table 1: Quality control analysis (Cayman Chemicals) of drug stocks.

Batch #1 (original stocks):

Compound	Purity	Mass expected	Mass actual	Concentration
Lipoxin A4	98.1%	351.1	351.3	100.53 µg/ml
Protectin DX	100.0%	359.4	359.2	103.10 µg/ml
Resolvin D1	99.2%	375.3	375.9	103.61 µg/ml
Resolvin E1	97.4%	349.5	349.3	49.77 µg/ml

Batch #2 (replacement stocks):

Compound	Purity	Mass expected	Mass actual	Concentration
Lipoxin A4	99.4%	351.2	351.3	98.00 µg/ml
Protectin DX	100.0%	359.4	359.4	100.40 µg/ml
Resolvin D1	99.2%	375.3	375.9	103.61 µg/ml
Resolvin E1	100.0%	349.5	349.2	49.98 µg/ml

To prepare the drugs for injection an amount is withdrawn from each stock vial using a calibrated glass micro-syringe (Hamilton; Reno, NV) to provide 25 µg/kg of material, as based on the rat's current body weight. Drug stocks are dispensed into brown glass Teflon screw cap vials and then dried down in ~ 20 min using a gentle stream of nitrogen gas. Residue is immediately dissolved by vortex mixer into 250 µl of sterile phosphate buffered saline, pH 7.2. Preparation of the drugs is begun within one hour prior to the start of injections and vials kept on wet ice to maintain compound stability. Drug solutions are hand warmed and drawn up into 1 ml syringes equipped with a 27G needle, less than 5 min prior to administration. Each drug is given (over 1 min) to the rats while under isoflurane anesthesia, by intravenous injection into the lateral tail vein (25 µg/kg; single bolus dose), within 10 min following blast wave exposure; and thereafter given every other day out to 14 days, for a total of 7 doses The booster shots are necessary to maintain the drug's plasma circulating and tissue uptake / incorporation levels as well as inflammation knock down status in the blast-injured retina and brain. Shams and blasted controls receive blank saline injections.

Results and Specific Conclusions:

During the study, we found that intravenous administration of the drugs into the rats by lateral tail vein injection was relatively easy and rapid to carry out. Typically, rats were injected with the drugs as soon as 5 min following blast exposure. Successful injection into the vein was demonstrated by ability to draw back blood, easy pushing of syringe plunger, and clearing of vein's blue coloration. There were only sporadic cases (> 2%) were entry into the vein was extremely delayed (> 5 - 10 min) or suspect with sub-dermal introduction the likely end result; thus, in our hands, delivery of these drugs into the blood stream was highly successful. Additionally, we did not see any outward indications of permanent vasculature damage to the tail by the repetitive injections, which could have caused chronic pain to the animals and thus negatively influenced their performance abilities on the visual discrimination behavioral test. Indeed, shams given repetitive saline injections in their tails exhibited normal home cage and visual discrimination testing behaviors (i.e., a strong resolve to earn food rewards). The drug treated blasted-rats, however, showed signs of loss of appetite and lethargy beyond those normally seen following blast, with protectin DX and resolvin D1 treatments early on displaying the worst symptoms. This was particularly noted during the visual discrimination testing (see Section IV), where the animal's physical activity (i.e., total lever presses) fell sharply soon after blast plus drug treatment (i.e., by 2 days-post) and never gained full recovery to baseline values. In contrast, blasted controls, often showed an apparent increase in test activity that peaked at 7 days post-injury (1.4-fold), as a way of trying to compensate for losses in visual function (i.e., attempting more guesses).

As a side experiment, we did a pilot group of two drug treated shams (i.e., one each for protectin DX and resolvin D1) to address the likelihood of drug toxicity side effects. Their ERG, visual discrimination, and histopathology results will be detailed in the respective sections below (i.e., III, IV, and V). We also submitted terminal blood (14 days post-blast) from these rats to the WRAIR department of Clinical Pathology for complete blood count (CBC) and blood chemistry panel work ups. The analysis was done to look for drug toxicity towards the white blood cells, kidneys, and liver. It is well known that these drugs work by suppressing the functions of white blood cells involved in exacerbating the inflammation of injured tissues, e.g., infiltration and cytokine release. Thus, we wanted to see if the drug treated rats had become ill, due to over suppression of white blood cells (i.e., immuno-suppressed). The CBC tested for levels of platelets, red blood cells, and the white blood cells - neutrophils, lymphocytes, monocytes, eosinophils, and basophils. The blood chemistry panel tested for levels of albumin, alkaline phosphatase, alanine transaminase, aspartate transaminase, bilirubin, cholesterol, creatinine, creatine kinase, electrolytes (e.g., Na^{+}, K^{+}, and Ca^{+2}), glucose, lactate dehydrogenase, total protein, triglycerides, and urea nitrogen. The test results were judged against reported normal physiological ranges for rats. All rats had greatly elevated glucose (2-fold) and slightly diminished albumin (30%) levels, which could simply be signs of metabolic stress from euthanasia. The drug treated shams showed no abnormalities in the levels of all blood cell types or other biochemical markers, especially those of kidney and liver function (e.g., aspartate transaminase and urea nitrogen, respectively). This implies the drugs are not harming the immune system or major organs. Interestingly, both animals had decreased levels (30%) of creatine kinase, a marker of proper muscle function; and this might explain some of their noted lethargy symptoms, but the pharmacology behind this is unknown.

III. **Electroretinogram (ERG) Recordings of Sham and Blasted Rats**

Materials and Methods:

Rats are adapted in full darkness for at least 6 hours, prior to being ERG tested. The dark adaptation is done to prime the retina light signaling responses and reduce retinal neuron background noise. Rats are then placed under anesthesia using isoflurane gas and pupils dilated using drops of tropicamide and phenylephrine (cholinergic antagonist and α-adrenergic agonist, respectively). The rat's eyes are also numbed with drops of propracaine. The animal, while maintained on gas anesthesia through a nose cone, is placed on a thermal blanket and a ground electrode fixed to the tail and reference electrodes to both cheeks, using short sub-dermal pins. Recording electrodes are attached to each cornea by placing the fine silver wire leads under contact lens affixed with methylcellulose solution. The rat is laid prone

with its face fully inserted into the light stimulus dome of a Handheld Multispecies electroretinogram unit (HMs-ERG; Ocuscience, Inc.). The eyes are then given a scotopic ERG exam (i.e., dark adapted response), using a light stimulus program that exposes the eyes to a series of white light flashes of six increasing intensities (i.e., 100, 300, 1000, 3000, 10,000, and 25,000 mcd.s/m^2), with each repeated 1 - 4 times (averaged) at an interval of 10 sec and a duration of 5 msec, and having a ramp spacing of 30 - 60 sec. This program was recommended to us by the manufacturer, for obtaining reliable ERG results on rats (i.e., a broad-range flash response curve). ERG responses arising from each eye are recorded simultaneously by computer and the peak voltage amplitudes of the underlying a- and b-wave forms and their implicit times (i.e., delay from zero to peak) are derived to judge the functional status of the retina photoreceptors and bipolar / amacrine neurons, respectively. After the ERG exam, to protect their dilated eyes from bright light damage, the rats are kept in darkness for at least several hours until they are recovered from anesthesia and pupil constriction reflex is restored; and then they are returned to their normal housing cages under standard lighting conditions. Rats are given an ERG exam at 1 day prior to blast over pressure wave exposure to establish their baseline light stimulus responses, and then retested once at 1, 7, and 14 days afterwards.

Results and Specific Conclusions:

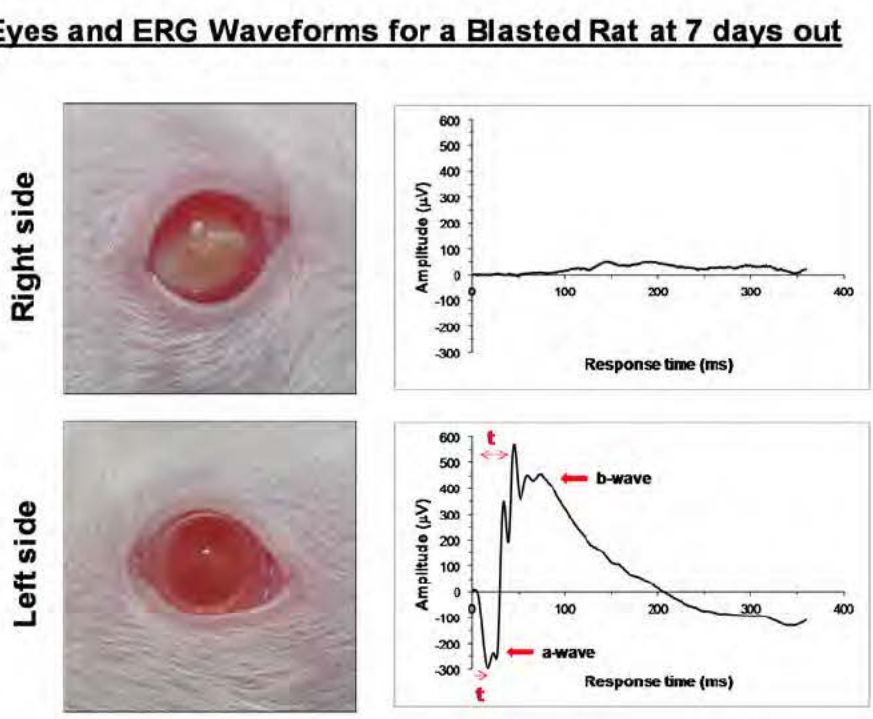
During the injury characterization phase of the study, we successfully carried out scotopic ERG recordings on a total of 14 sham and 15 blasted rats (see supplemental Figure A) at 1 day prior to injury (baseline) and then at 1, 7, and 14 days thereafter. Early on, we had some problems with the isoflurane anesthesia used to sedate the rats during the procedure and lost a few animals due to respiratory or cardiac failure. In order to make the ERG data easier to present, only the peak amplitudes and implicit times for the resulting a- and b-wave responses at the light flash intensity of 3000 mcd.s/m^2 were plotted out versus time post-blast. This flash intensity is recommended by the International Society for Clinical Electrophysiology of Vision (ISCEV) as an optimal light stimulus for doing ERG recordings in research animals and humans (McCulloch, 2015).

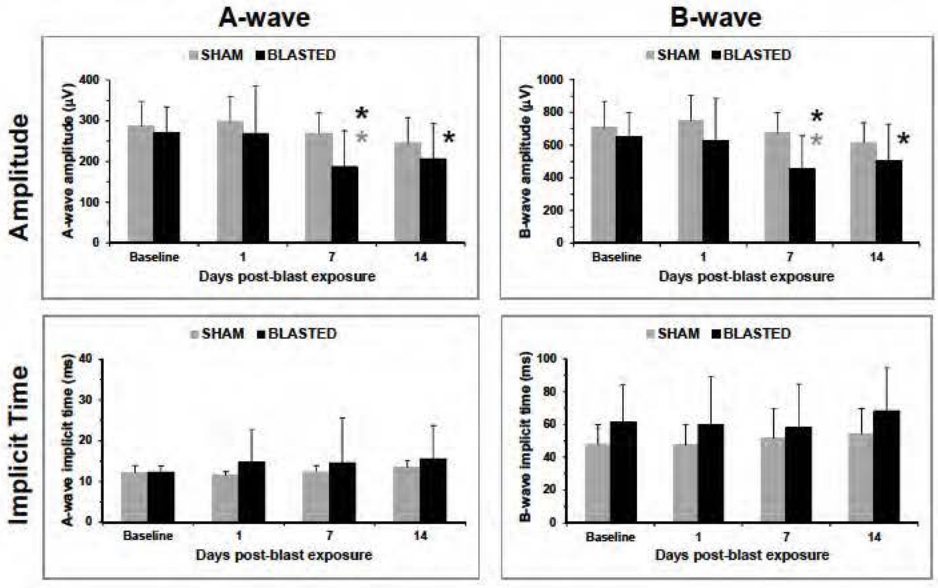
Shown in below in Figure 3 are the eyes and ERG traces for a blasted alive-rat examined at 7 days out. The right eye shows marked corneal scarring and little light signaling response. Necropsy of this animal at 14 days post-blast showed the right eye to be atrophied with severe retinal degeneration. The left eye's exterior shows some redness and ERG waveform's amplitudes and implicit times appear normal, except the b-wave has oscillatory spikes that are suggestive of neuron misfiring; but at 14 days post-blast the external and retinal pathology of this eye was normal. Next in Figure 3 are shown the bar graphed ERG amplitudes and implicit times for right and left eyes of shams (gray) versus blasted (black) animals (mean ± SD; n = 14 and 15) at baseline and then 1, 7, and 14 days following exposure.

We found that the right eyes of blasted rats had significant decreases in a- and b-wave amplitudes at 7 days post-exposure when compared to its baseline and sham values (31, 30, 30, and 33%, respectively), and at 14 days post-exposure versus only its baseline (24 and 22%, respectively); but no differences were seen at 1 day out. These findings strongly indicate there is substantial blast induced retinal injury on the right side, which faces the shock wave. In contrast, for this group, the left eyes had no pronounced differences in ERG responses; where there was only a minor significant decrease in a-wave amplitude at 14 days post-blast (13%). This indicates negligible functional impairment is occurring to the left eye following blast, which is consistent with it being opposite to the oncoming shock wave. While ERG amplitudes were decreased in the blasted rats, there were no significant differences for both eyes detected for a- and b-wave implicit times at any day post-exposure, implying the retinal deficits are likely due to neuronal cell death (e.g., photoreceptor losses), as opposed to impairment of signaling rate in living cells. The modest differences detected here between sham and blasted rats are not due in part to high same animal variability in the retina light responses from resting state, since we found that dark adapting the rats overnight (16 hours) versus 6 hours prior to the ERG exam did not enhance or further stabilize the amplitudes or implicit time for the resulting waveforms (n =6; data not shown). Also, we found that back to back ERG reruns done on some animals to verify results gave nearly identical readings. There were some concerns that the ERG testing itself could lead to disturbances in retina function due to factors such as repetitive exposure to the light flash stimulus. Shams, however, were not found to significantly decline in ERG amplitudes or implicit from baseline out to 7 and 14 days of testing;

neither was there an apparent trend at 7 days for this phenomenon. It should still be taken into consideration that the ERG procedure may exacerbate any blast wave injuries to the eye and thus should be kept to a minimum as much as possible.

Figure 3: ERG amplitudes and implicit times of sham and blasted rats.





Left Eyes

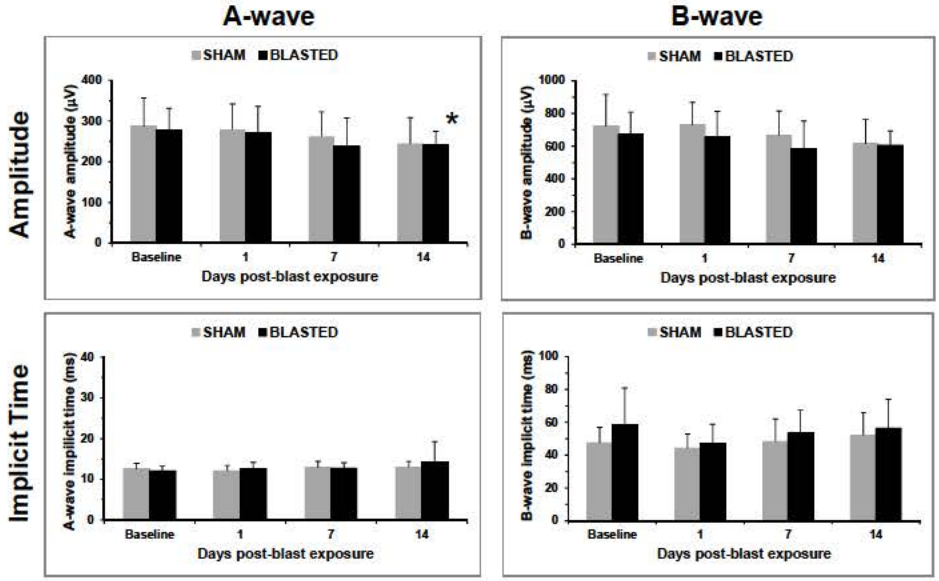


Figure 3. Top panel; eyes and ERG waveforms for a representative blasted rat at 7 days out; the right eye shows marked corneal scarring and little retinal signaling response. Left eye's exterior and ERG trace are relatively normal. The a- and b-waves of the ERG trace are indicated by red arrows; t = implicit time. Bottom panels: bar graphs of ERG amplitudes and implicit times for sham versus blasted animals (mean ± SD; n = 14, 15), as taken at baseline and then 1, 7, and 14 days post- blast exposure. Baseline recordings were all done at 1 day prior to blast. Light flash stimulus used here was 3000 cd.s/m^2. Separate panels are shown for right and left eye responses. *$p \leq 0.05$ vs. blasted baseline; *$p \leq 0.05$ vs. shams, as carried out by simple t-test.

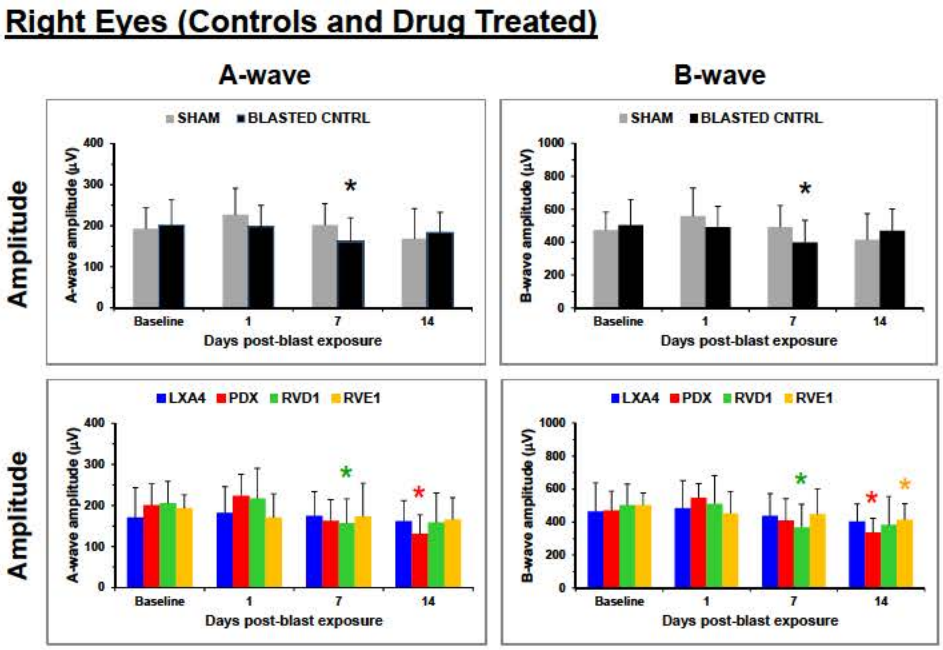
During the experimental drug testing phase of the study, we successfully carried out scotopic ERG recordings on 10 shams, 22 blasted controls, and 12 lipoxin A4, 11 protectin DX, 12 resolvin D1, and 12 resolvin E1 drug treated blasted-rats (see supplemental Figure A) at 1 day prior to injury (baseline) and at 1, 7, and 14 days thereafter. Two additional shams were similarly treated with protectin DX or resolvin to look for any drug toxicity side effects towards ERG outcomes. As before, to make the ERG data easier to present, only peak amplitudes at the light flash intensity of 3000 mcd.s/m2 were plotted out versus time post-blast. We also line graphed the data out as a percentage of baseline values, so that small changes over time are more easily visualized and interpreted.

Shown below in Figure 4 are the bar graphs of ERG amplitudes for sham (gray), blasted control (black), and drug treated blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and RVE1 = resolvin E1 / orange) (mean ± SD; n = 10, 22, 12, 11, 12, and 12, respectively), as taken at baseline and then 1, 7, and 14 days post-blast. Panels are separately shown for right and left eyes. Below each set of bar graphs is the corresponding b-wave data represented in line graphs as a percentage of the baseline recordings. The apparent efficacy order for the drugs is shown above each of these graphs. As found previously, ERG a- and b-wave amplitudes of both the right and left eyes of blasted controls (n = 22) showed a significant decrease from baseline by 7 days post-exposure (25%), which was not seen for the shams (n = 10). Detection of a left eye deficit this time is likely due to the higher powering of the group sizes, and caused by a through head-propagation or wraparound of the blast waves. For both eyes, however, we did not detect a significant difference this time from sham values, which may be due to under powering of this group. We still believe that the blast exposure is causing a moderate retinal injury primarily on the side facing the insult. When examined for changes from baseline, as well as compared to shams and blasted controls, we found that for the right eyes the drugs

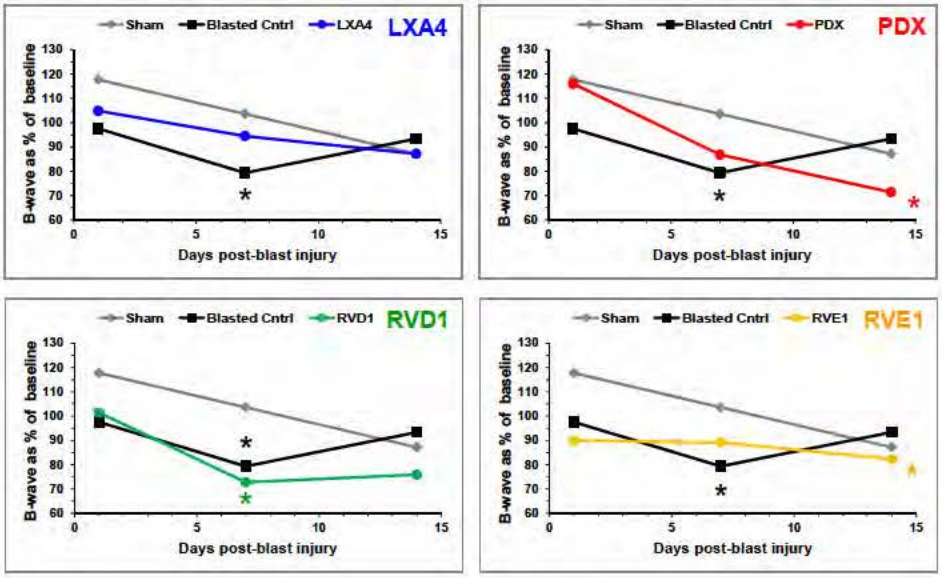
showed an apparent efficacy order of LXA4 > RVE1 ≈ PDX ≈ RVD1. Only, in the case of lipoxin A4 treatments there was an absence of significant declines out to 14 days post-injury. In contrast, resolvin E1, protectin DX and resolvin D1 were significantly decreased (15 - 30%) by as early as 7 days out. Similarly, the left eye showed that lipoxin A4 followed closely by protectin DX were the most efficacious; whereas, the other two drugs had significant losses (10 - 25%) by 14 days out (i.e., LXA4 ≈ PDX > RVE1 > RVD1), again implying that they are not as neuro-protective against retinal cell dysfunction and/or degeneration post-blast. The relatively minor dissimilarities seen in the drug efficacy order for the right versus left eyes are likely due to differences in directional nature of the retinal injuries, with the right eye that bore the brunt of the blast wave being the more reliable indicator of drug treatment effectiveness.

Shown below in Figure 5 are the bar graphed ERG amplitudes for normal shams (gray) and shams that were drug treated (PDX = protectin DX / red and RVD1 = resolvin D1 / green) (mean ± SD; n = 10, 1, and 1, respectively), as taken at baseline and then 1, 7, and 14 days post-blast. We found that the ERG a- and b-wave amplitudes of the right and left eyes for both drug treated shams (i.e., protectin DX and resolvin D1) showed a trend to decrease in value versus baseline out to 14 days post-exposure (30 - 40%). This suggests that these drugs are causing some negative side effects toward retinal neuron function; however, the biochemical mechanism is uncertain. Consistent with this, the normal shams (non-treated) did not show any marked declines in ERG function over the same time period.

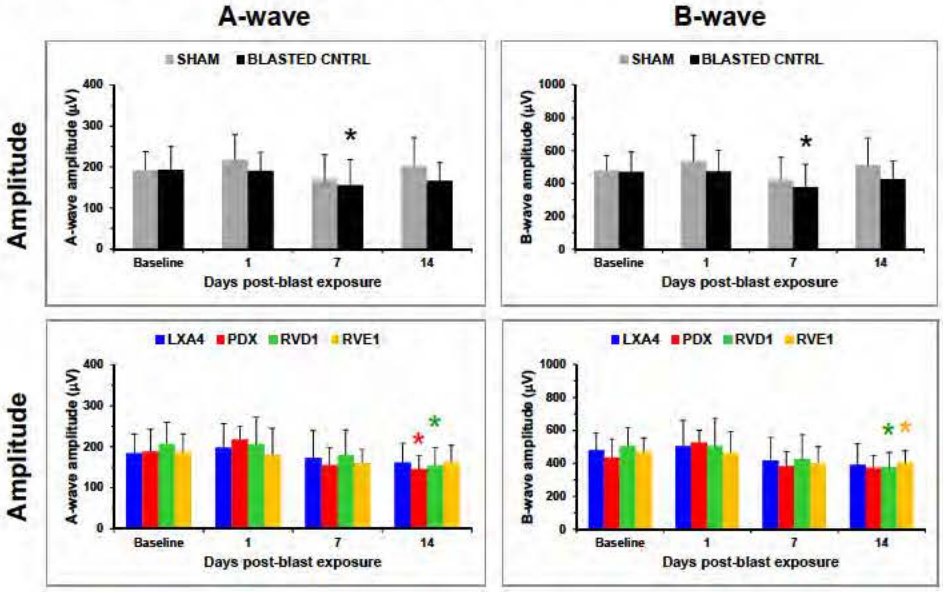
Figure 4: ERG amplitudes of sham, blasted control, and drug treated blasted-rats.



Efficacy Order: LXA4 > RVE1 ≈ PDX ≈ RVD1



Left Eyes (Controls and Drug Treated)



Efficacy Order: LXA4 ≈ PDX > RVE1 > RVD1

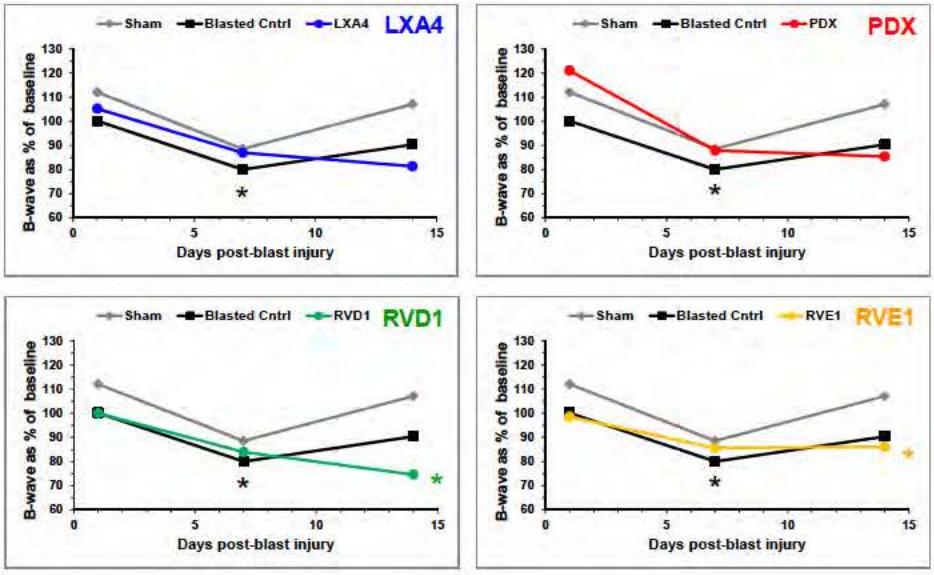
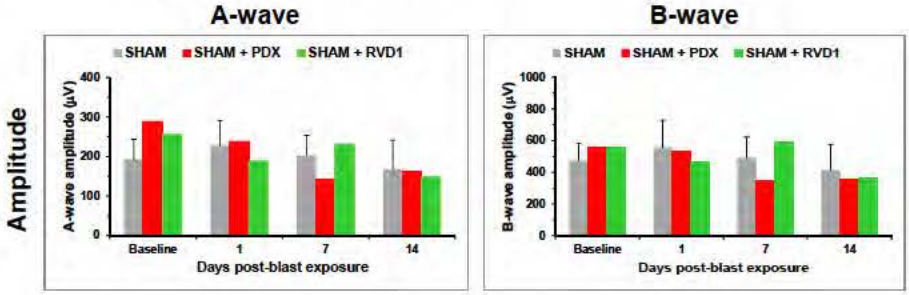


Figure 4. Bar graphs of ERG amplitudes for sham (gray), blasted control (black), and drug treated blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and RVE1 = resolvin E1 / orange) (mean ± SD; n = 10, 22, 12, 11, 12, and 12, respectively), as taken at baseline and then 1, 7, and 14 days post-blast. Baseline recordings were all done at 1 day prior to blast. Light flash stimulus used here was 3000 cd.s/m^2. Panels are separately shown for right and left eyes. Below each set of bar graphs is the corresponding b-wave data represented in line graphs as a percentage of the baseline recordings. The apparent efficacy order for the drugs is above each of these graphs. *$p \leq 0.05$ vs. baseline values. Statistical analysis was done by simple t-test.

Figure 5: ERG amplitudes of normal sham and drug treated sham rats.

Right Eyes (Normal and Drug Treated Shams)



Left Eyes (Normal and Drug Treated Shams)

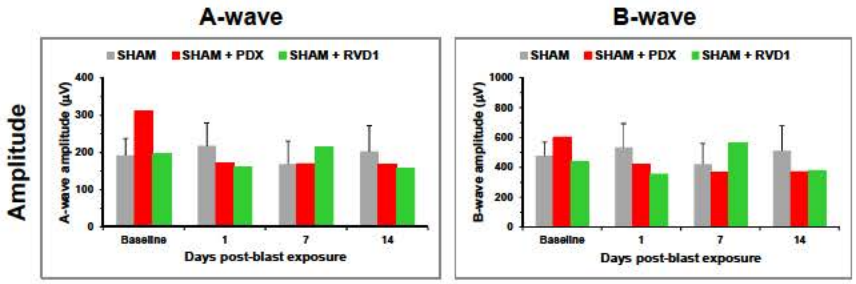


Figure 5. Bar graphs of ERG amplitudes for representative normal shams (gray) and shams treated with drugs (PDX = protectin DX / red and RVD1 = resolvin D1 / green) (mean ± SD; n = 10, 1, and 1, respectively), as taken at baseline and then 1, 7, and 14 days post-blast. Baseline recordings were all done at 1 or 3 days prior to blast. Light flash stimulus used here was 3000 cd.s/m2. Panels are separately shown for right and left eyes. There were no statistical differences found for the normal shams from baseline, as carried out by simple t-test. Statistical comparisons were not carried out for the drug treated shams, due to only single animals being done for each group.

IV. Visual Discrimination Testing of Sham and Blasted Rats

Materials and Methods:

Animals are placed inside visual discrimination conditioning boxes (Med Associates, Inc.), consisting of a standard housing cage that is equipped with a response lever, a cue light mounted above the lever, a water bottle, and a recessed food trough connected to a dispenser capable of discharging small pellets of standard rodent chow. The boxes also have an internal house light, which is continually left on during the animal's entire stay inside. Training the animals for the vision test consists of a sequence of three individual program phases presented to the rats over four sessions. For the initial training session, rats are placed in the conditioning boxes for a 12 hour overnight period. This session consists of two phases. The first phase simply cycles the cue light on and off in conjunction with extending the response lever out and in. The aim is to draw the rat's attention to the lever and get it to press the lever while out and the cue light is on. During each trial, the cue light and lever stay active for 30 sec. Pressing the lever during this time rewards the animal with a food pellet treat. If the lever is not pressed during the active period, a timeout occurs. The cue light goes off and the lever temporarily retracts for a time period (inter-trial interval) randomly chosen between 10 and 30 sec in 5 sec increments. In phase 1, however, a free food pellet is issued every 20 min to help stimulate the rat. After 100 correct lever presses in phase 1, the program moves to the second phase, where the lever is always left in the extended position while the light cycles on and off and free food pellets are not issued. Again, the goal here is to achieve 100 correct lever presses only while the cue light is on.

The second training session is also a 12 hour overnight session that begins with phase 2 (or a phase 1 repeat, if necessary). The active cue light / lever period here is reduced from 30 to 15 sec, and again no free food pellets are given. After 100 correct trials, the program moves onto a phase 3 in which a punishment is introduced when the rat incorrectly presses the lever while the cue light is off. During this phase and all later testing, the punishment consists of turning off the boxes' house light and retracting the lever for 15 sec. The animal then goes on to training sessions 3 and 4 that utilize a 2 hour time period each with no limit on earned food pellets (correct responses), when running phase 3. These two sessions are meant to reinforce the concept of depressing the lever while the cue light is on and reduce the amount of guessing (i.e., depressing the lever when the cue light is off). For these sessions, the active cue light / lever period is reduced to 8 sec. A correct response accuracy of at least 60% at the end of the training (session 4) is our absolute criterion for the animals to move forward into actual visual capacity testing following blast exposure. We do not have a clear explanation for failure of some rats to learn the test, other than they may be simply uninterested in the food pellet rewards or overly anxious of the test

environment. While we do not continue on with visual discrimination testing of non-performing rats, they are retained as sham or blasted animals and then subjected to ERG recordings and histopathology, as scheduled in the project.

Finally, baseline visual discrimination tests are performed for successfully trained rats on the day prior to and in the morning directly before blast wave exposure (days 8 and 9, respectively). In these tests, the program runs through a scrambled order of cue light intensity levels with random inter-trial intervals as described above until 117 trials (9 at each of 13 cue light levels) have been completed. For our scale, each cue light level is a highly diverse reduction (4 - 80%) in intensity of the previous one, ranging from maximum brightness down to near zero output. Using a photometer we determined that the exact intensities of the cue light under full darkness are 0, 0.05, 0.3, 0.6, 0.9, 1.6, 2.0, 3.4, 4.6, 5.8, 6.0, 10.3, and 13.8 lux. Standard room lighting is typically 100 - 150 lux, making visualization of the cue light at our chosen settings, to some extent, challenging for the rat. At 2, 5, 7, 12, and 14 days following blast wave exposure, the rats are retested against the randomized light intensities for 2 hours. Number of correct responses / food pellets earned (i.e., pressed the lever only when the cue light was on) will be used to determine the animal's visual capacity threshold. We have tried to design the task to be an acuity test as opposed to purely a memory test.

Results and Specific Conclusions:

During the injury characterization phase of the study, we successfully carried out visual discrimination testing on a total of 11 sham and 10 blasted rats (see supplemental Figure A) in the morning prior to injury (baseline) and then at 2, 5, 7, 12, and 14 days thereafter. In order to attain these group sizes, we ran a total of 29 rats through visual discrimination testing; however, only 21 of those were fully carried on for 14 days past the initial training phase due to inability to master the task (28% failure rate). For the most part, rats that failed to be adequately trained appeared to be uninterested in obtaining the food rewards, even though we always limited their food intake (3 - 4 pellets) the night before the test as a motivational tool. Animals that flunked out were still used as shams or blasted rats for ERG and histopathology assessments.

Shown below in Figure 6 are the bar graphed visual discrimination responses (i.e., lever presses with a cue light to earn a food reward) for sham (gray) versus blasted (black) animals (means ± SD; n = 11 and 10, respectively) at baseline and then 2, 5, 7, 12, and 14 days following blast wave exposure. Baseline responses were those recorded in the morning directly before blasting. While the two groups did not significantly differ at any time point for total, correct, and incorrect lever responses, there was a trend over time, peaking at 7 days post-injury, for the blasted rats to have a higher number of total and incorrect responses compared to shams (1.3 and 1.6 - fold; p =0.24 and 0.20, respectively). Interestingly, the blasted rat's correct responses trended to decrease from baseline values by 2 days out (28%; p = 0.15), which was not seen in the shams. In light of the small group sizes used, we tried relaxing the stringency for the statistics from a two to one tailed t-test, but this still did not achieve significance for any of these parameters in the blasted rats (p = 0.12, 0.10, and 0.08, respectively). These trends, however, lead to the reasonable speculation that the blasted rats are perhaps simply "guessing" more during the test to earn a similar quantity of food rewards (e.g., frequently hitting the lever at random).

Overall, our visual discrimination test findings are consistent with the degree and timing of those we found for the ERG recordings, i.e., peak deficits (~30%) at 7 days post-blast with substantial recovery signs at 14 days. We also carried out Pearson's correlation analysis between the ERG amplitudes (a- and b-wave; right eye) and visual discrimination correct lever responses both at 7 days (n = 10, each; graph not shown) and found there was not a significant relationship between the two (r = -0.57 and -0.50; p = 0.09 and 0.12, respectively). This finding is consistent with the lack of significant differences for the visual discrimination test, but may also indicate any deficits are due to factors besides retina damage, such as unrelated brain dysfunction (e.g., memory and learning deficits). Blast injured rats could also be using other senses to work around the test (e.g., hearing cue light relay switches activate), thus damping any differences due to vision loss.

Figure 6: Visual discrimination test responses for sham and blasted rats.

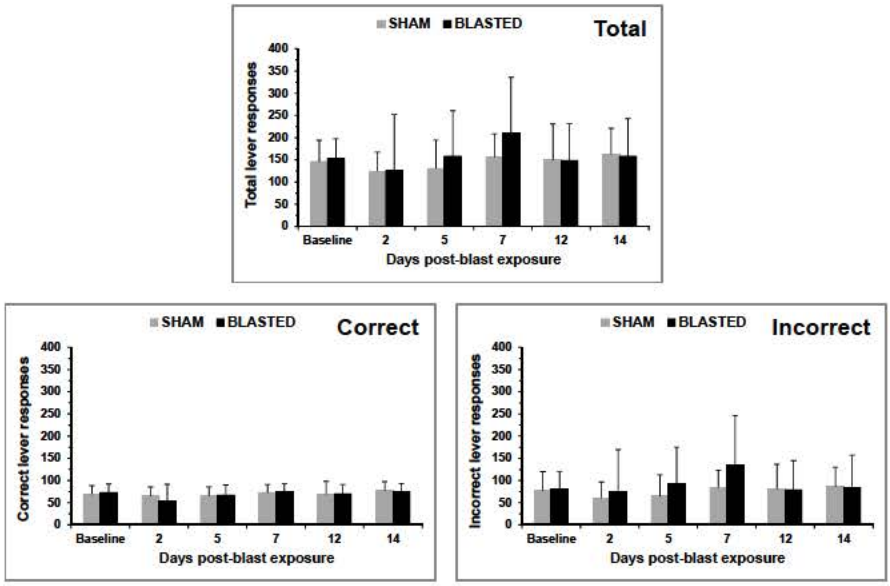


Figure 6. Bar graphs of total, correct, and incorrect lever responses (i.e., lever presses in accordance with a cue light to earn food rewards) for shams (gray) versus blasted (black) animals (mean ± SD; n = 11 and 10, respectively), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast. Baseline was done in the morning before blasting. No significant differences were found on any parameter.

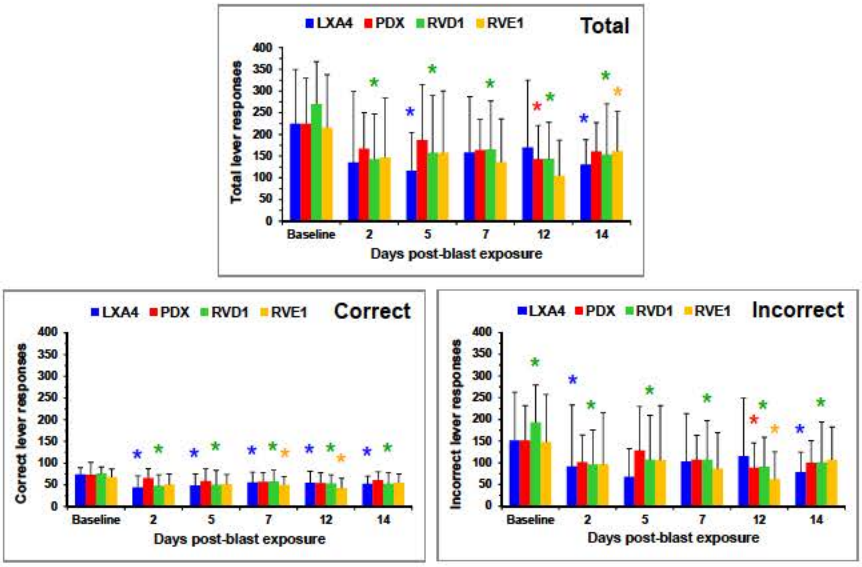
During the experimental drug testing phase of the study, we successfully ran a total of 67 rats through visual discrimination testing out to 14 days post-blast. The training dropout rate with our starting animals was near to what we found during the injury characterization phase of the study (i.e., 31%). In this case, all rats that mastered the test were preferentially reserved for drug treatments post-blast, and thus sham and blasted controls were limited in numbers. Animals that completed the visual discrimination testing consisted of 12 lipoxin A4, 11 protectin DX, 12 resolvin D1, and 12 resolvin E1 treated blasted-rats (see supplemental Figure A). We managed to also obtain 11 blasted controls; 6 shams that underwent no other procedures (naïve shams); a sham treated with drugs (protectin DX and resolvin D1), as a toxicity study; and one blasted animal treated with protectin DX that had completely lost its right eye and incurred slight damage to the left eye (blinded PDX). This latter animal was kept aside and served as an excellent positive control to judge if the test could pick up permanent vision deficits. The right eye was ruptured during the blast; and thus, was surgically enucleated the same day. The left eye had, at 7 days post-blast, an ERG of 94 and 272 μV (a- and b-waves, respectively) and at 14 days a retina histopathology score of 3 / mild (data not shown).

Shown below in Figure 7 are the bar graphed visual discrimination responses (i.e., total, correct, and incorrect lever presses in accordance with a cue light to earn a food reward) for drug treated blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and RVE1 = resolvin E1 / orange) (mean ± SD; n = 12, 11, 12, and 12, respectively), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast. Also shown in graphs below these are the correct responses for each drug as a percentage of their baseline values (line graphs). Baseline responses were recorded in the morning directly before blasting. The apparent drug efficacy order is shown above these latter graphs. It is extremely difficult to draw conclusions on a drug's efficacy by simply looking at the number of lever responses the rats made during the test. As with the ERG results, representing the data as a percentage of baseline values allowed any changes over time to be more easily visualized and interpreted. We found by correct responses there was an apparent drug efficacy order of PDX > RVE1 > LXA4 ≈ RVD1. Protectin DX was the most efficacious and did not change from baseline values out to 14 days. In

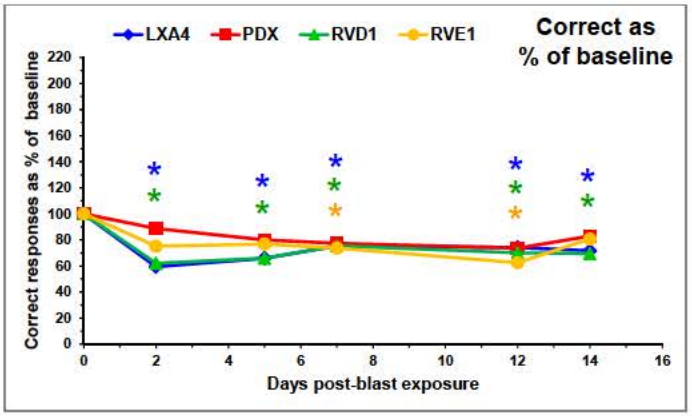
contrast, the other 3 drugs had significantly decreased (25 - 40%) by 7 days post-injury. Unlike here, lipoxin A4 was previously found to be the most efficacious by ERG recordings. This again suggests the visual discrimination test performance may be influenced by things other than retinal degeneration status.

Overall, the largest drop in correct responses for the drug treated animals occurred by 2 days post-blast. As shown below in Figure 7, we examined the number of correct responses attained under each cue light setting (i.e., intensity), at this specific time point post-injury. This was done to see if there were any differences in the threshold at which the drug treated rats could detect the cue light. Surprisingly, we found that all animals had very low cue light intensity thresholds of 0.3 - 0.6 lux, which were significantly higher (2-fold) than the control setting of zero illumination. This value is close in illumination to that of a full moon or deep twilight of 0.1 and 1 lux, respectively (www.engineeringtoolbox.com/light-level-rooms-d_708.html); which makes sense, since rats are well suited for nocturnal vision. To make the test more accurate, we could have used a finer scale up to an illumination of 2 lux or less. Regardless, the amount of correct responses significantly above zero found across all cue light intensities (0.05 - 13.8 lux) for each drug confirms the efficacy order that we had found above (i.e., PDX > RVE1 ≈ LXA4 > RVD1).

Figure 7: Visual discrimination test responses for drug treated blasted-rats.



Efficacy Order: PDX > RVE1 > LXA4 ≈ RVD1



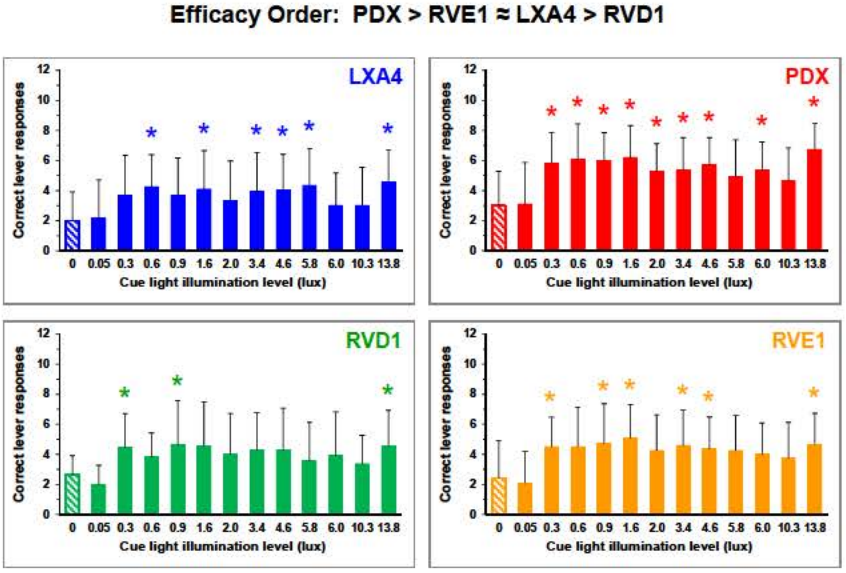


Figure 7. Bar graphs of total, correct, and incorrect lever responses (i.e., lever presses in accordance with a cue light to earn a food reward) for drug treated blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and RVE1 = resolvin E1 / orange) (mean ± SD; n = 12, 11, 12, and 12, respectively), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast. Also shown in the two graph panels below these are the correct responses as a percentage of their baseline values (line graph) and the correct responses attained under each cue light setting (lux) at 2 days post-blast (bar graphs). Baseline was done in the morning before blasting. The apparent drug efficacy orders are shown above each graph. *p < 0.05 vs. baseline or zero illumination (striped bar) values. Statistical analysis was done by simple t-test.

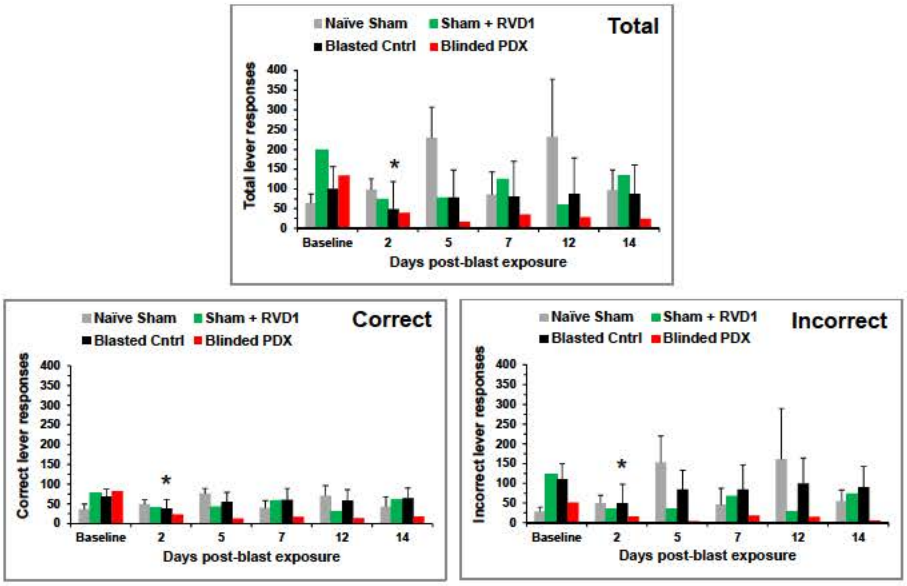
Shown below in Figure 8 are the bar graphed visual discrimination responses (i.e., total, correct, and incorrect lever presses in accordance with a cue light to earn a food reward) for naïve shams (gray); a sham treated with drugs (RVD1 = resolvin D1 / green); blasted controls (black); and a drug treated blasted-rat that was fully blinded in the right eye (red; blinded PDX) (mean ± SD; n = 6, 1, 11, and 1, respectively), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast. Also shown in graphs below these are the correct responses for each drug as a percentage of their baseline values (line graphs), with the apparent drug efficacy order located above. We found that the naïve shams continually improved in their correct responses, being significantly above baseline (2-fold) by 5 days afterwards. This is in line with the absence of other invasive tests (e.g., ERG) being done to the animals, which could detrimentally effect their "mood" to perform the task. Consistent with this, for the drug treated sham (resolvin D1) there was a decrease in values compared to baseline over 14 days post-blast (21 - 61%). We saw similar losses for a sham given protectin DX (data not shown). Blasted controls showed a comparable pattern of overall response deficits versus baseline (6 - 47%), with significantly less correct responses by 2 days out (47%). This is still likely due in part to injuries to the eyes, since the drug treated blasted rat missing its right eye (blinded PDX) had severe declines in correct responses over all days examined (72 - 84%); implying it had great difficulty seeing the cue light, and therefore the test works.

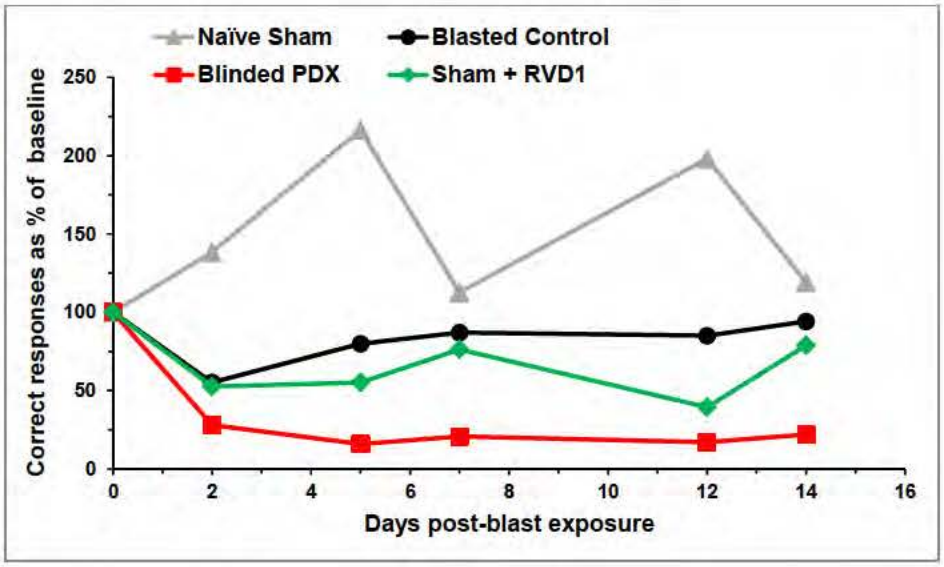
As also shown in Figure 8, to confirm our results, we examined the number of correct responses attained at each cue light setting, specifically at the peak deficit of 2 days post-injury. Naïve shams had a threshold of 0.3 lux and remained significantly above a setting of zero at all illuminations thereafter. While the drug treated sham (resolvin D1) distinctly struggled to see the cue light at several settings (4.6, 5.8, and 6.0 lux), it had an identical threshold to the naïve shams with values of the same range at all other illuminations. Blasted controls had a threshold shifted to 0.6 lux and failed to significantly discern the cue light at two setting thereafter (1.6 and 10.3 lux). Likewise, the drug treated rat missing its right eye had a threshold of 0.6 lux and obtained very low correct responses (0 - 3) at all cue light intensities. We

previously found in the injury characterization phase of the study that blasted controls (n = 10) did not have significant declines in baseline responses, but instead had a trend to make more "guesses" (total and incorrect responses) that peaked at 7 days post-blast.

In general, our findings demonstrate that the drug treatments alone can disrupt the ability of the rats to perform the visual discrimination task; however, a biochemical mechanism for this is uncertain. As an aside (data not shown), we examined for each of the drug treatment groups the percentage of correct out of total lever responses (% correct) on each day and found they did not decline from baseline values, and even trended to markedly increase for a sham given protectin DX by 12 days out (2-fold). The majority of rats tested here have similar % correct responses to what we previously found for shams and blasted controls in the first year of the study (~ 50%). These findings suggest the drug treated shams haven't really lost their underlying ability to accomplish the test; but instead are less eager to perform it, possibly due to visual field disturbances or simply general malaise. Unfortunately, this still makes it difficult to judge in blasted rats that are treated how much of any remaining deficits in correct responses are due to the injury alone, as opposed to experimental side effects; and thus, seriously undermines the desirability for the visual discrimination tests use in future studies.

Figure 8: Visual discrimination test responses for naïve shams, a drug treated sham, blasted controls, and a drug treated partially blinded rat.





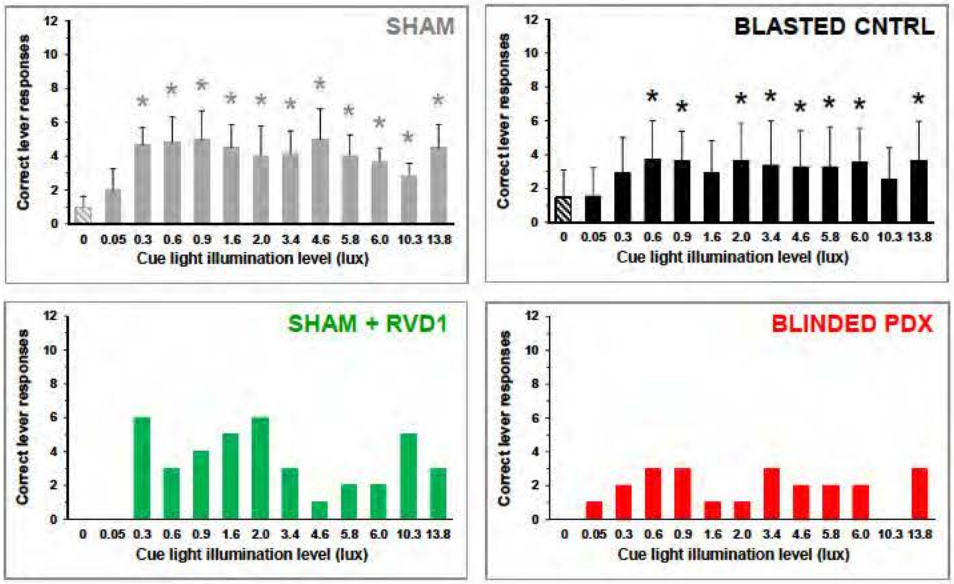


Figure 8. Bar graphs of total, correct, and incorrect lever responses (i.e., lever presses in accordance with a cue light to earn a food reward) for naïve shams, a shams treated with drugs (PDX = protectin DX / (RVD1 = resolvin D1 / green), blasted controls (black), and a and a drug treated blasted-rat that was fully blinded in the right eye (red; blinded PDX), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast (mean ± SD; n = 6, 1, 11, and 1, respectively), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast. Also shown in the two graph panels below these are the correct responses as a percentage of their baseline values (line graph) and the correct responses attained under each cue light setting (lux) at 2 days post-blast (bar graphs). Baseline was done in the morning before blasting. *p < 0.05 vs. baseline or zero illumination (striped bar) values. Statistical analysis was done by simple t-test. Statistical comparisons were not carried out for the drug treated sham and drug treated partially blinded rat, due to only single animals being done for each group.

V. Histopathology of Eyes and Brains from Shams and Blasted Rats

Materials and Methods:

At 14 days post-blast wave exposure, after a final visual discrimination test and ERG exam, rats are euthanized for tissue collection. Animals are anesthetized with isoflurane and then perfused transcardially with saline, resulting in euthanasia by blood exsanguination, followed by 4% paraformaldehyde saturated with picric acid. Prior to saline perfusion, a blood sample is taken by cardiac puncture. Liver lobe is also collected and quick frozen on dry ice. Blood is later spun to obtain the plasma fraction. Plasma and liver are stored frozen at -80^{o}C for use by other investigators in our lab. After perfusion, whole brain and eyes are removed; and observational notes and pictures are taken to record the gross external pathology. Tissues are then subjected to further processing over several days with other fixative reagents. Brains are washed in sucrose solution. Eyes are post-fixed, to harden the globes, with isopropanol, trichloroacetic acid, zinc chloride, and ethanol. Fixed eyes and brains are sent out to FD NeuroTechnologies, Inc. (Ellicott City, MD) to be made under a contract agreement into slides containing cross sections stained with hematoxylin and eosin / H&E (eyes and brain) and silver (brain only), to hunt for signs of neuronal apoptosis as indicated by cell morphology disturbances and axonal fiber tract degeneration, respectively. Eyes are cut in a single horizontal section (5 µm) through the pupil's central axis. Brains are cut in 11 evenly-spaced vertical sections (30 µm) through the cerebrum, to cover all underlying visual centers. Prepared slides are examined under an axial light microscope equipped with an image capture camera and a computer having image processing software. For the brains, neurons in visual centers known to be effected by blast injury are assessed on the slides (e.g., optic tract, optic chiasm, superior colliculus, geniculate nucleus, and occipital cortex). For the eyes, distinct neuronal layers making up the retina are examined (e.g., ganglion, bipolar / amacrine, and photoreceptor cells). Injuries are assigned relative damage scores, using a rank scale of 1 - 6 (e.g., none, slight, mild, moderate, severe, and catastrophic), as judged by consensus of one to two "blinded" reviewers (lab technicians) and one "un-blinded" moderator (senior scientist) who advises on regions of interest (e.g., artifacts versus injury) and settles score split decisions.

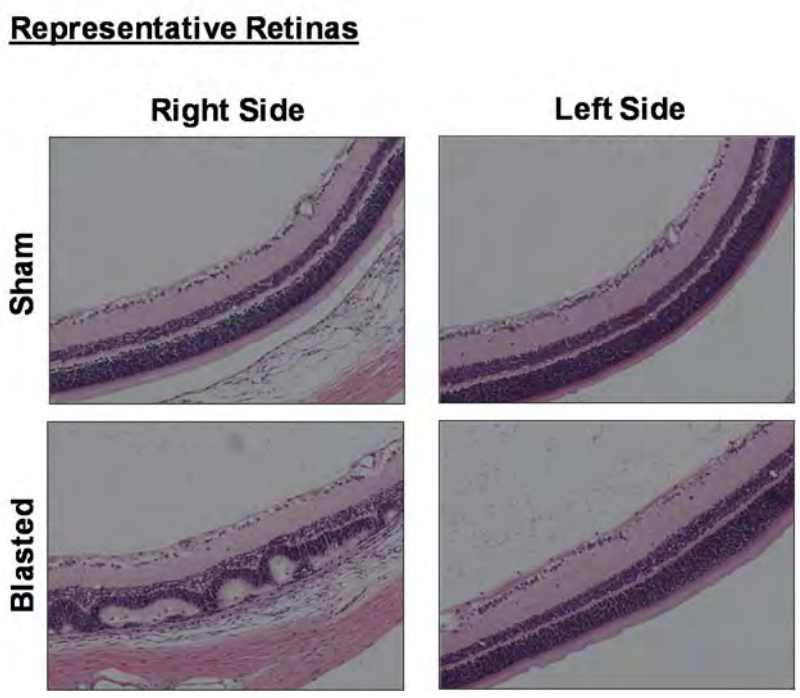
Results and Specific Conclusions:

During the injury characterization phase of the study, we submitted eyes (right and left pairs) and brains from 14 shams and 15 blasted rats, collected at 14 days post-injury, for histopathology processing by an outside contract company (see supplemental Figure A). The eyes and brains were made into H&E (eye and brain) and silver (brain only) stained microscope slides, returned to us, and then put through relative damage scoring (rank scale of 1- 6) for neuronal cell damage to the retina and brain visual centers (e.g., optic tracts). Shown below in Figure 9 are representative microscope images for the right and left side retinas and brain optic tract and superior colliculus regions (20x and 4x magnifications, respectively) of sham and blasted rats. Retinas are the dark purple ribbon "like" structure with distinct neuronal cell body layer divisions. Brain optic tracts are the dark brown oval "like" structure sandwiched between two cerebral cortex lobes; whereas, the superior colliculi are the muffin "like" structures that sit atop the mid brain. The retinas and brain regions of the sham are free of obvious cellular perturbations. The right retina of the blasted rat, however, shows marked reorganization and degeneration of the photoreceptor and bipolar / amacrine cell layers. Correspondingly, the right and left optic tracts and superior colliculi of the same animal show black staining consistent with axonal fiber tract degeneration. The left optic tract and superior colliculus shown here are more intensely stained than the right side, which may be explained by the optic nerve fiber bundles from the retinas in rats switching hemispheres by 90% after the optic chiasm. This implies that much of the brain axonal degeneration is coming from loss of afferent signaling input from the right retina (i.e., anterograde degeneration). However, the presence of marked axonal degeneration on both sides of the brain could also indicate that some of the neuronal damage is from the blast wave displacing these visual processing regions alone.

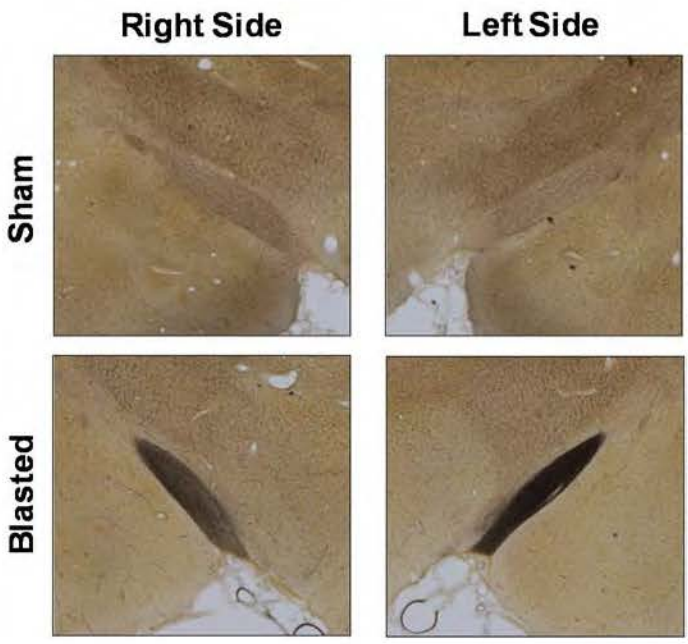
Also shown below in Figure 9 are bar graphs for the relative damage scores (rank scales shown; see insets) of the retina and brain optic tracts for shams (gray) and blasted (black) rats (n = 14 and 15, respectively); where for this type of data, all statistical analyses were done using the appropriate Mann-Whitney U, two-way (i.e., paired), analysis of variance test. We found significantly more neuronal cell

damage (2-fold) to be present in the right retinas, but not for the left, and in right and left optic tracts to the same degree (3 and 3-fold, respectively). The right retina and right and left brain optic tract injury incidence (mild or greater) was 67, 53, and 67%, respectively, as based on these scores. We could have also scored the superior colliculus and other interconnected brain visual centers. This data could also be verified with cell body counts and layer thickness for the retina and silver staining optical densities for the brain. Limitations to these additional measures, however, are defining the specific regions of interest to assess for the retina and finding a consistent background to subtract from the brain optical densities. Overall, the retina and brain relative damage scores found here strongly support our current contention that blast wave exposure leads to a double component injury to the visual system, which is likely a combination of direct retinal cell layer damage, anterograde degeneration of brain visual pathway nerve fiber bundles (i.e., retina to optic nerves to optic chiasm to optic tracts), and direct axonal shearing of brain regions. It is also possible for directly damaged brain axons to stimulate degeneration backwards into the retina (i.e., retrograde degeneration) through loss of efferent signaling input.

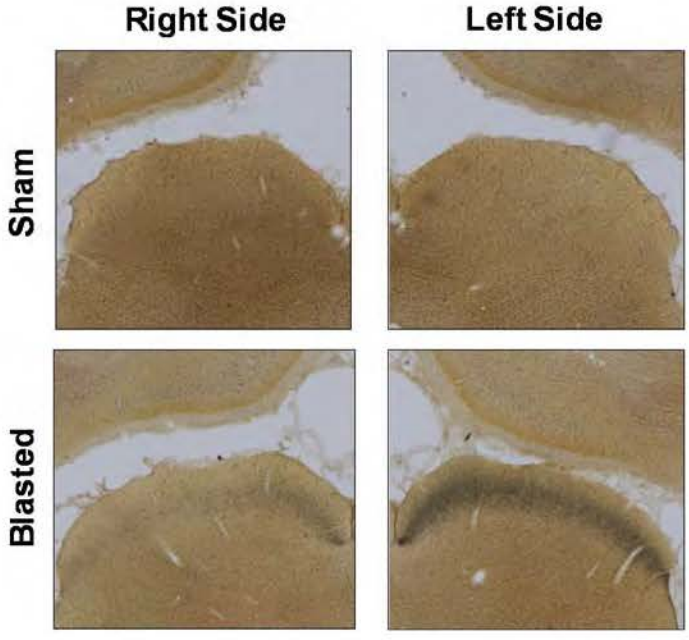
Figure 9: Retina and brain injuries and relative damage scores for blasted rats.



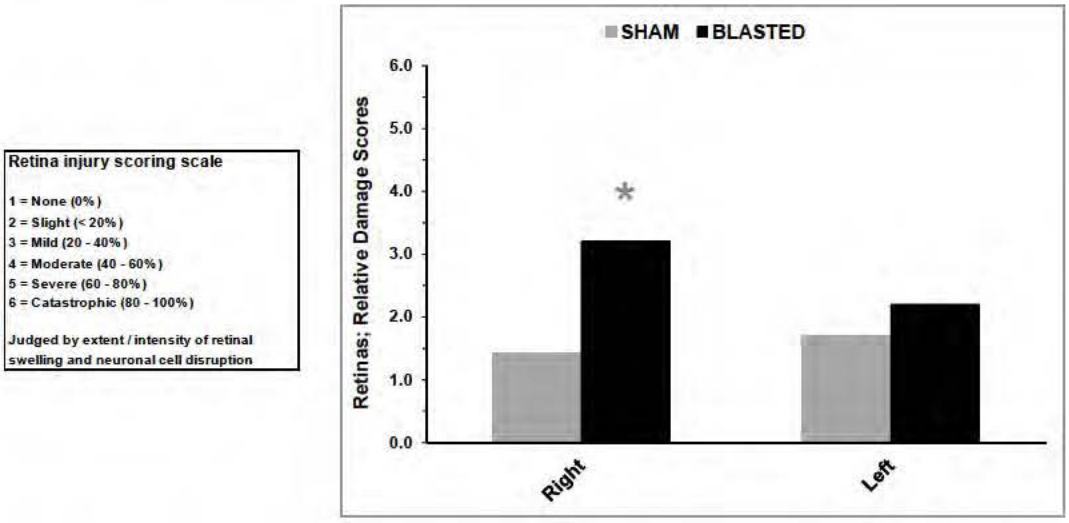
Representative Brain Optic Tracts



Representative Brain Superior Colliculi



Retina Relative Damage Scores



Brain Optic Tract Relative Damage Scores

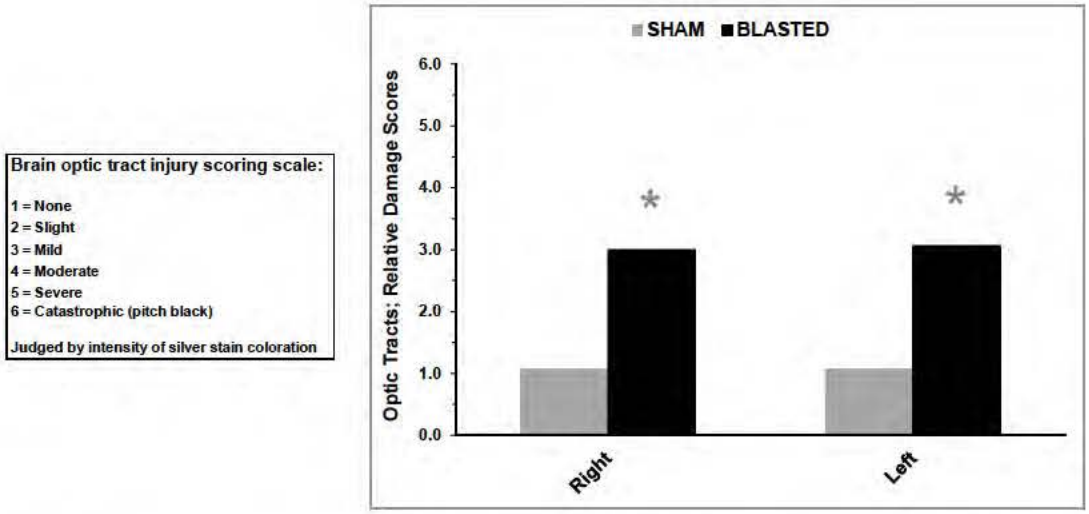
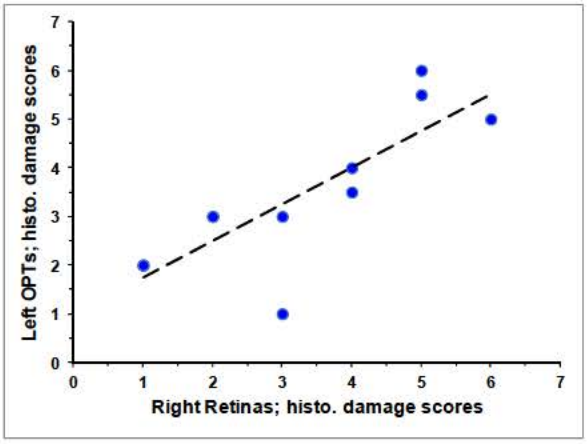


Figure 9. Top 3 panels; representative microscope images for cross sections of retinas and brain optic tracts and superior colliculi from sham and blasted rats at 14 days post-injury, stained with hematoxylin and eosin (H&E) and silver, respectively. Blasted retina sections (20x) show extensive neuronal cell layer degeneration to be present on the right side. Likewise, blasted brain sections (4x) show the presence of marked axonal fiber tract degeneration (black coloration) on both the right and left sides. Bottom 2 panels; bar graphs of relative damage scores for retinas and brain optic tracts of sham (gray) and blasted (black) rats (n = 14 and 15, respectively). Rank scales (1 - 6) used for scoring are shown in detail in the left insets. *$p \leq 0.05$ vs. shams. Statistical analysis was done by Mann-Whitney U test for non-parametric data. Likewise, standard deviations are not given, as is appropriate for this type of data.

As a post-hoc comparison, for some of these rats (n = 11), we carried out Pearson's correlation analysis between the retina and brain histopathology results and those of the ERG (amplitudes) and visual discrimination test (correct responses). For simplicity of conclusions, for the most part, only the results from the right eyes or retinas and their signal input corresponding left optic tracts were used for these comparisons. As shown below in the scatter plots of Figure 10, we compared right retina and left optic tract relative damage scores (n = 11, each) and found there was a highly significant positive relationship between the two (r = 0.81), despite the brain's left side being contra-lateral to the blast wave impact. In contrast, relative damage scores for the right retinas and right optic tracts did not correlate (graph not shown; r = 012; p = 0.73). This greatly supports our histopathological evidence that blast wave injury to the retina is leading to anterograde axonal degeneration of the opposing brain visual centers.

As shown below in Figure 10, we then compared the right eye ERG amplitudes (a- and b-wave) at 7 days post-blast to right retina and left optic tract relative damage scores (n = 11, each) and found they all had significant negative relationships between each other (r = - 0.76, - 0.72, - 0.77, and - 0.78, respectively). This implies that the ERG deficits we saw in the blasted rats are a direct measure of retina signaling function; and helps eliminate other potential causes, such as cornea or lens damage. However, when comparisons were made using the ERG data at 14 days post-blast, we did not find correlations with retina and optic tract relative damage scores (graphs not shown; r = - 0.25, p = 0.45; r = - 0.30, p = 0.36; r = - 0.24, p = 0.48; and r = - 0.32, p = 0.33, respectively). This finding is not surprising, since the ERG deficits showed signs of substantial recovery at 14 days post-blast. Finally, we compared the visual discrimination test correct responses at 7 days post-blast and right retina and left optic tract relative damage scores (n = 7, each) and found none of these correlated with each other (graphs not shown; r = 0.38, p = 0.40; and r = 0.31, p = 0.50, respectively). Again, this finding is not surprising, since none of the visual discrimination test results were significantly different between shams and blasted rats; and the ERG amplitudes did not correlate with this data either.

Figure 10: Pearson's correlation analysis for ERG versus histopathology results.



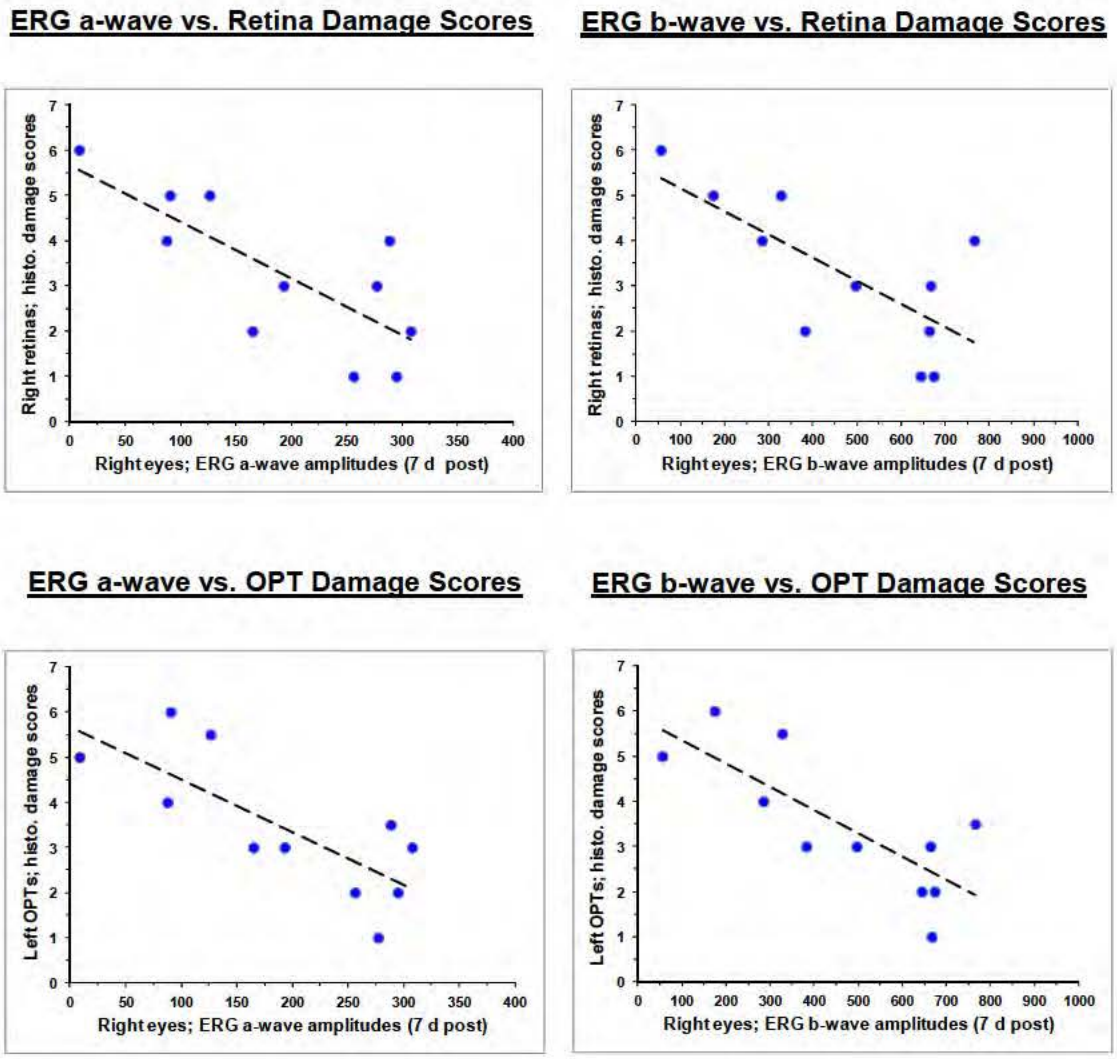


Figure 10. Scatter plots for Pearson's correlation analysis between right retina and left brain optic tract (OPT) relative damage scores (n = 11, each); right eye ERG amplitudes (a- and b-wave) at 7 days post-blast and right retina relative damage scores (n = 11, each); and right eye ERG amplitudes (a- and b-wave) at 7 days post-blast and left brain optic tract (OPT) relative damage scores (n = 11, each). Significant relationships ($p < 0.05$) were found for all comparisons (left to right and top to bottom: $r = 0.81$, - 0.76, - 0.72, - 0.77, and - 0.78, respectively).

During the experimental drug testing phase of the study, we submitted eyes (right and left pairs) and brains from 11 shams, 22 blasted controls, and 12 lipoxin A4, 11 protectin DX, 12 resolvin D1, and 12 resolvin E1 drug treated blasted-rats, collected at 14 days post-injury, for histopathology (see supplemental Figure A). Two of these shams were treated with protectin DX or resolvin D1 to look for any evidence of drug toxicity side effects. One normal sham that fully underwent ERG testing out to 14 days died from respiratory problems shortly after the procedure, and thus the eyes and brain did not get collected for histopathology. As before, eyes and brains are made into H&E (eye and brain) and silver (brain only) stained microscope slides, returned to us, and then the retinas and brain optic tracts assigned relative damage scores.

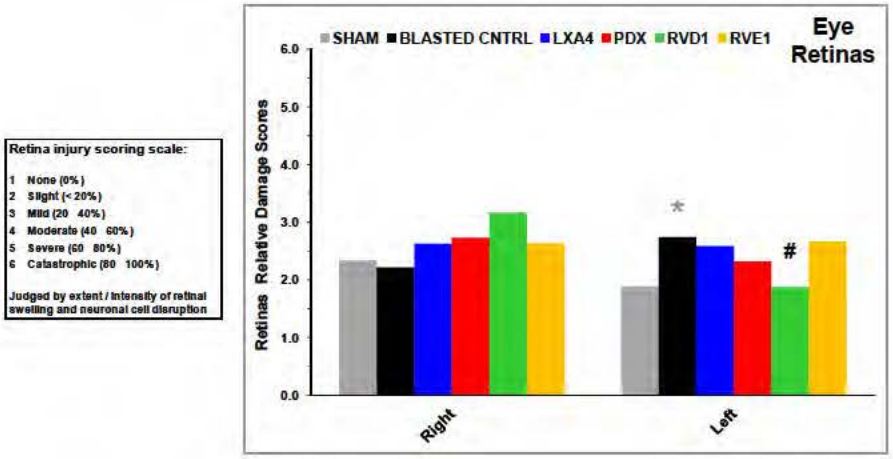
Shown below in Figure 11 are the bar graphed relative damage scores (rank scale is shown; see inset) for the right and left retinas and brain optic tracts of sham (gray), blasted control (black), and drug treated blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and RVE1 = resolvin E1 / orange) (n = 9, 22, 12, 11, 12, and 12, respectively for both) at 14 days-post blast. Shown below these are the distribution graphs for the raw data points along with their mean bars, which is the appropriate way to provide the variances associated with non-parametric data, as based on rank scale scores. The apparent drug efficacy orders are found above these graphs. As before, all statistical analyses were done using the Mann-Whitney U, two-way, analysis of variance test.

When compared to sham values, for both the right and the left retinas, the drugs showed an apparent efficacy orders of LXA4 ≈ RVE1 ≈ PDX > RVD1 and RVD1 > PDX > LXA4 ≈ RVE1. Consistent with this, lipoxin A4 proved to be the most efficacious drug during ERG testing of the right eyes. The histopathology findings, however, are based mainly on trends in the averages and single point distributions (i.e., extent above the mean) of the damage scores; except for the left retina, the shams and resolvin D1 treated blasted-rats were significantly lower than the blasted controls (31 and 32%, respectively). In contrast, we previously found that blasted controls only had significantly more (2-fold) right-side retina damage versus shams (n = 14 and 15, respectively). Lack of significant right retina injuries in the blasted controls here was due in part to some higher than usual damage scores for the shams. Retinal injuries in the shams, while mild at the greatest on our scale, could be coming from rough or over touching of the eyes during the ERG exams. Alternatively, we are using a non-pigmented strain of rats in our studies (i.e., Sprague Dawley), which are notoriously susceptible to retinal scarring from chronic and/or bright light exposure. Likewise, after ERG exams the rat's pupil constriction reflex is still weakened, by residual effects of the dilation drugs, which greatly elevates the chances of retina injury from just the holding room lights.

When compared to sham values, for the left brain optic tracts, the drugs showed an apparent efficacy order of LXA4 > RVD1 > PDX ≈ RVE1, respectively, again as mainly based on trends in the damage score averages and single point distributions. The left optic tract is directly innervated with the axons from right retina, which faced the blast; since in rats, the connecting optic nerves switch fiber direction by 90% at the optic chiasm. All of the drug treatments, however, showed left side damage scores that were significantly higher when compared to shams (2 - 3-fold). Blasted controls for the left side were also significantly higher than the shams (2 - 3-fold). None of the drug treatments, however, had left side damage scores that were lower than the blasted controls. Despite this shortcoming, the estimated drug efficacy order of the left optic tract partially agrees with that of its corresponding right retina in that lipoxin A4 was ranked the highest in both cases. The right optic tract, which mainly receives axonal connections from the left retina, showed a drug efficacy order of RVD1 > PDX > RVE1 ≈ LXA4. Again, all of the drug treatments were significantly higher than the shams (2 - 3 fold) and not significantly different from the blasted controls; however, there was a distinct trend for resolvin D1 and protectin DX (11 and 26%, respectively) to be lower than the blasted controls In this case, the drug efficacy order of the right optic tract was very similar to that of its corresponding left retina. At least for lipoxin A4, the efficacy order of the right retina and left optic tract, is similar in nature to that obtain for the ERG testing of the right eyes. Caution must be taken, however, when interpreting the drug efficacy results for both optic tracts, since the orientation of the blast wave to the eyes was asymmetrical (i.e., right side on) and there is a small degree of axonal cross talk between the optic tracks originating within the optic chiasm. Over all, our histopathology findings point to a very slight protection, if any, against blast-induced neuronal degeneration being afforded by the drugs to the retina and brain visual centers.

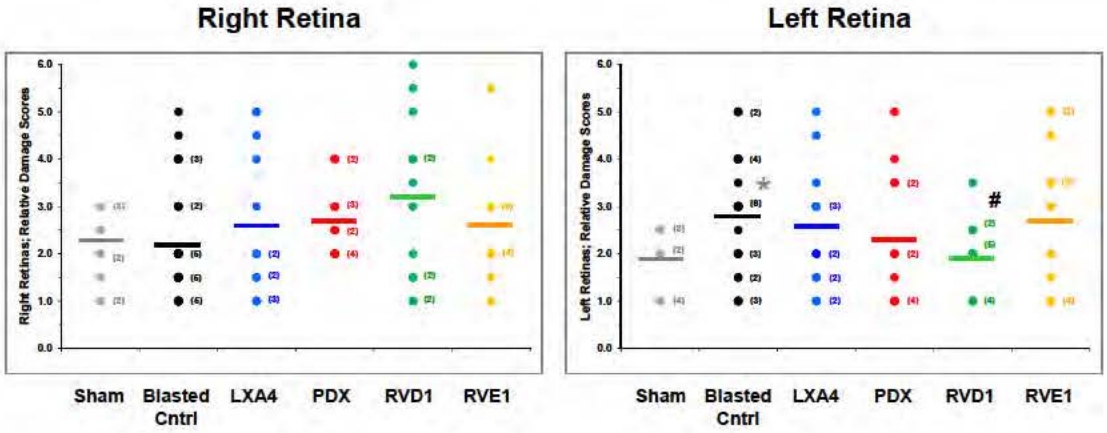
Figure 11: Retina and brain optic tract relative damage scores for shams, blasted controls, and drug treated blasted-rats.

Retina Relative Damage Scores

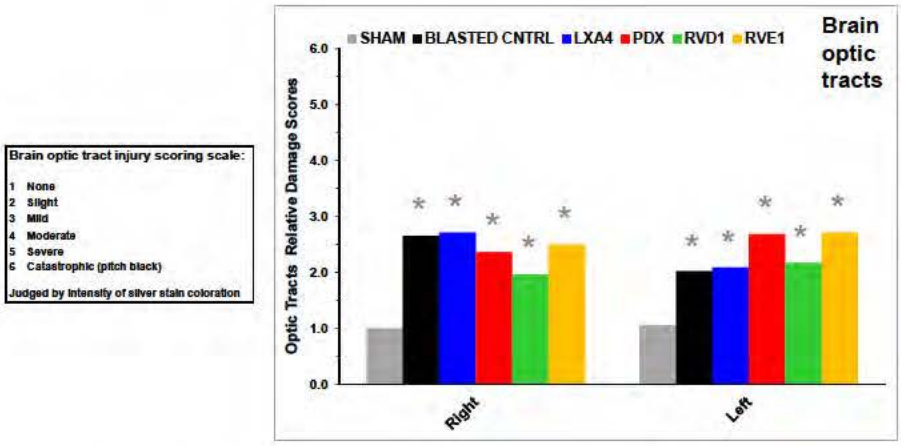


Efficacy Order Right: LXA4 ≈ RVE1 ≈ PDX > RVD1

Efficacy Order Left: RVD1 > PDX > LXA4 ≈ RVE1



Brain Optic Tract Relative Damage Scores



Efficacy Order Right: RVD1 > PDX > RVE1 ≈ LXA4

Efficacy Order Left: LXA4 > RVD1 > PDX ≈ RVE1

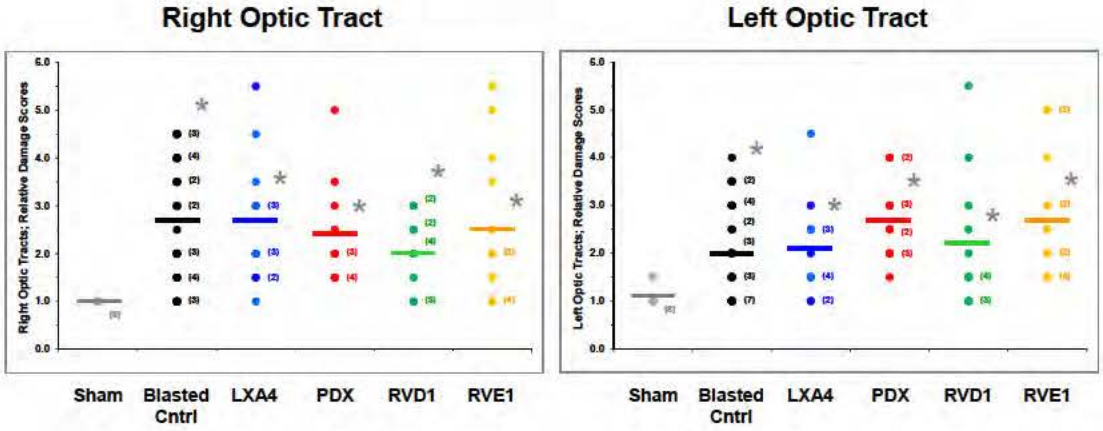


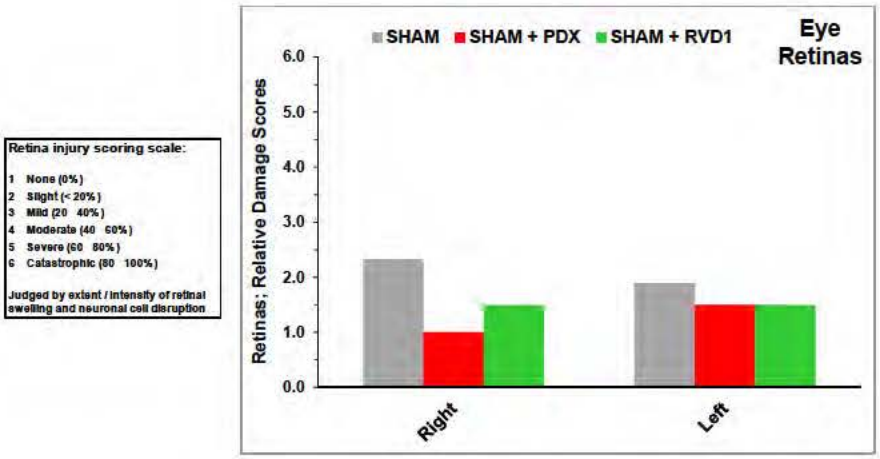
Figure 11. Bar graphs for relative damage scores of eye retinas and brain optic tracts (right and left) of sham (gray), blasted control (black), and drug treated sham or blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and and RVE1 = resolvin E1 / orange) (n = 9, 22, 12, 11, 12, and 12, respectively) at 14 days-post blast. Rank scales (1 - 6) used for scoring neuronal cell damage in each are shown in the left side insets. Shown below these are the distribution graphs for the raw data points along with their mean bars. Numbers in brackets show the amount of repetitive samples present (> 1) at each data point. *$p \leq 0.05$ vs. shams and $^{\#}p \leq 0.05$ vs. blasted controls. Statistical analysis was done by Mann-Whitney U test for non-parametric data. Likewise, standard deviations are not given, as is appropriate for this type of data.

Shown below in Figure 12 are the bar graphed relative damage scores (rank scales are shown; see insets) for the right and left retinas and brain optic tracts of normal shams (gray) and shams that were drug treated (PDX = protectin DX / red and RVD1 = resolvin D1 / green) (mean ± SD; n = 9, 1, and 1,

respectively for both) at 14 days-post blast. Overall, the right and left retinas and brain optic tracts of both drug treated shams showed no impelling signs of neuronal cell degeneration, in that their damage scores were not significantly greater than those of the normal shams, thus helping rule out this possibility for the cause behind the drug's apparent negative side effects towards visual function in the rats. Indeed, for the right retinas, the protectin DX and resolving D1 treated shams trended well below the normal shams (57 and 36%, respectively), suggesting they may have been shielded, if anything, by these drugs.

Figure 12: Retina and brain optic tract relative damage scores for normal shams and drug treated shams.

Retina Relative Damage Scores



Brain Optic Tract Relative Damage Scores

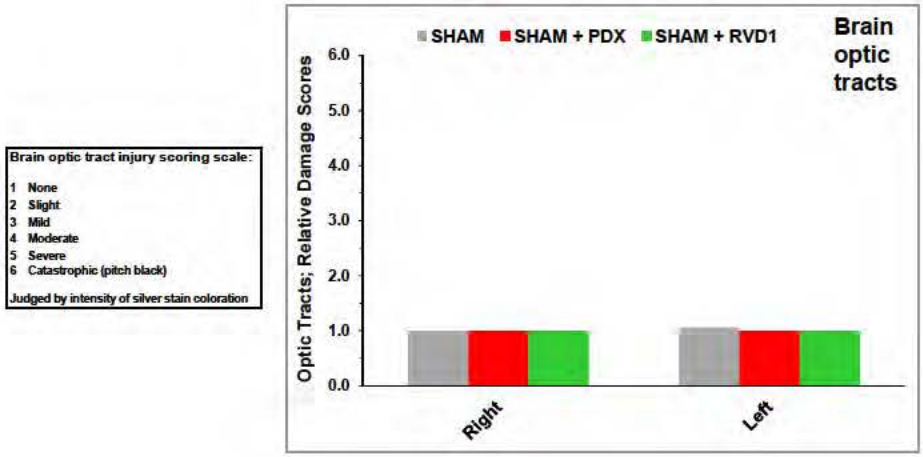


Figure 12. Bar graphs for relative damage scores of retina and brain optic tracts (right and left) of normal shams (gray) and drug treated shams (PDX = protectin DX / red; and RVD1 = resolvin D1 / green) (n = 9, 1, and 1, respectively) at 14 days-post blast. Rank scales (1 - 6) used for scoring neuronal cell damage in each are shown in the left side insets. Statistical comparisons against normal shams were not carried out for the drug treated shams, due to only single animals being done for each of these two groups.

KEY RESEARCH ACCOMPLISHMENTS

1) Using a compressed air driven shock tube, established a rat model of blast over pressure wave induced neuronal injuries to the eyes (retina) and brain visual centers. This model produces closed-injuries to the retina and brain consistent with those suffered by soldiers subjected to explosions in the field of operations. This is of great interest, since others have carried out eye injury studies using extremely poor simulations of blast wave exposure (e.g., point blank air blasts from a modified paint ball gun) and thus, maybe reporting inaccurate data regarding characterization and drug treatment of the resulting neuronal cell degeneration.

2) Using electroretinography (ERG), we showed that blast wave exposure in rats by 7 days out leads to significant disruption (30% less) in the retinal signaling response to a light flash stimulus. By analysis of the resulting ERG waveforms, the deficits appear to involve all neuronal cell layers of the retina, including the photoreceptors that initiate the visual transduction process. This is of great interest, since others have reported that the only the outer most ganglion cell layer of the retina that connects to the optic nerve is damaged by blast wave exposure; and thus, should be the primary target for drug interventions. Photoreceptor protein enhancing medications, however, merit consideration.
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3) Using visual discrimination behavioral testing, we showed that blast wave exposure in rats by 2 to 7 days out leads to significant disruption (30% less) in the ability to recognize a variable cue light and then correspondingly press a lever to earn food rewards. While there is a likely an underlying memory component to this test, analysis of correct lever responses suggested an increase in the threshold for the observable cue light intensity is occurring. This is of great interest, since others have not looked for deficits in visual related behavioral function following blast wave exposure.

4) Using histopathology, we showed that blast wave exposure in rats by 14 days out leads to significant neuronal cell degeneration in the retina and brain visual centers (2 - 3 fold increase). The damage encompasses most of the visual pathway including the photoreceptors along with other retinal cells and extends into the brain from the optic tracts back to the superior colliculus, ending just before the occipital cortex. This is of great interest, since others have not reported a presence of deep brain involvement with retinal damage following blast wave exposure; and it likely results from anterograde degeneration of the retina and interconnected axons prior to the brain's optic chiasm (i.e., retinal ganglion cells and optic nerve). Thus, this implies that drug interventions must also target the brain to be effective at protecting visual function following blast.

5) Using the above outcome measures, we showed that intravenous injection of "naked" metabolites of omega-3 and -6 polyunsaturated fatty acids - that are unknown to be pro-resolving mediators of inflammation - immediately following blast wave exposure in rats was not substantially efficacious out to 14 days afterwards at preventing neuronal cell degeneration in the retina and brain visual centers. Only one of the four drugs tested, lipoxin A4, generated any plausible improvements in visual function post-blast, but as found on ERG testing alone. This is interesting, since others have recently reported that this compound and the resolvins D1 and E1 that we tested are markedly neuro-protective in rodent models of traumatic brain injuries, as generated by focal point contusions (i.e., controlled cortical impact and lateral fluid percussion). This suggests that these drugs, as well as other candidates, are tissue pathology specific and blast wave induced neurotrauma (i.e., diffusive axonal shearing) may require advanced delivery platforms (e.g., nanoparticles) to effectively target the widespread retinal and brain injury sites.

CONCLUSIONS

Over the course of this study, using techniques of electroretinography (ERG), visual discrimination behavioral testing, and histopathology, we have conclusively shown in rats that a single exposure to a blast over pressure wave, by 7 days out, leads to retinal signaling dysfunction with neuronal cell damage (e.g., photoreceptor degeneration) as the underlying cause. This in turn, we found this apparently stimulated anterograde degeneration of axonal fiber tracts in the brain visual centers (e.g., optic tracts and superior colliculus), due to loss of retinal signaling input. It is known that traumatic injuries to the retina produce anterograde degeneration of axonal fibers feeding into the brain starting at the retina ganglion cell layer, but has been proven reversible with drug interventions (Thanos, 1991; Avilés-Tigueros, 2003). Some of the brain damage could also be the result of the blast wave directly impacting the nervous tissue. We exposed the rats to an intense blast wave (20 psi; 260 Hz) that produces mild to moderate traumatic brain injuries in the animals, making it a realistic scenario to what a soldier might experience in the field during attacks from explosive devices (Warden, 2006). Ocular tissues are extremely fragile, especially the retina, so can they be easily displaced and damaged by a blast wave as it is channeled into the skull's eye sockets. We realize that soldiers are issued protective goggles in the field, but blast induced eye injuries will always be of great risk due to potential non-compliance of wear, blast wave penetration, or being blown off the face (Lemke, 2013). Indeed, the incidence of closed eye injuries in blast exposed soldier is 43%, with 26% of these cases involving serious retina damage and long lasting impairments in vision (Cockerham, 2011; Capó-Aponte, 2012; Lemke, 2013). Our animal model had a similar externally notable closed eye injury incidence of 67%, and likewise 67% of the rats had internal retinal cell damage. This implies that the majority of blast wave exposed soldiers may suffer some degree of retinal injuries, especially if their eyes are not protected. Additionally, we found that the brain visual processing centers of the blasted rats were damaged to an incidence of at least 53%; which is something to our knowledge that has not been clinically investigated in blast injured soldiers as an underlying pathological component for subsequent problems with vision loss.

Retinal and brain visual center degeneration in rats has been previously produced by us and others using blast waves made by compressed air driven shock tubes, but was only proven by histopathology of the tissues (Petras, 1997; Koliatsos, 2011; Wang, 2014; Choi, 2015). Others have also observed retinal signaling deficits by ERG in conjunction with retinal cell damage by histopathology in mouse models of blast wave exposure (Hines-Beard, 2012; Jiang, 2013; Mohan, 2013; Bricker-Anthony, 2014a, b; Dutca, 2014; Bricker-Anthony, 2015); but the injury is unrealistically catastrophic (e.g., optic nerve avulsion) or delayed in manifestation (e.g., several months) due to very poor simulation of the blast waves. For example, multiple studies have fired a high velocity air rifle directly at a mouse's cornea (Hines-Beard, 2012; Jiang 2013; Bricker-Anthony, 2014a, b; Dutca, 2014; Bricker-Anthony, 2015) and another put mice inside an uncontrolled air expansion blast chamber having an obscure end delivery pressure (Mohan, 2013). These studies misleadingly report results of air jet and not blast wave models. Recently, a rat study set off live explosive charges hung near the caged animals (Zou, 2013); but, while this open air approach is a very authentic blast simulation, it is highly difficult to precisely reproduce the resulting blast wave that strikes the animals due to many influential factors (e.g., air humidity, charge size / shape, surface reflections, and incidence angle). In contrast, our model here utilizes high fidelity simulated air blast waves (i.e., Friedlander waveform) as generated in an environmentally sealed shock tube to induce the injuries; and thus, produces visual system damage of a more realistic degree and time post-exposure to the human condition.

We have recently started looking into the effects of blast injury to the visual system under variable conditions that might be encountered by soldiers in the field. Typically, we expose the rats to single blast waves in a right side on orientation, but have also examined what happens to the eyes and brain, if the rat is blasted face on. We found this positioning to produce less severe and consistent retina and brain optic tract injuries over time, which may be due to the blast wave channeling around the rat's stream lined nose. We have also applied repetitive blast exposures to the animals (i.e., double blast at a 1 min interval) and found that this produces highly aggressive retina degeneration with extensive brain visual center involvement. Recent collaborative efforts by us, with the Pittsburgh NMR Center for Biomedical Research at Carnegie Mellon University, have shown there are extensive structural deformations,

accompanied by the infiltration of macrophages, in the retinas and brain visual centers of rats within several days following double blast exposure (Foley, 2013; Calabrese, 2014). Improvement of our blast model in the future may also include looking at the visual system injury effects over a wide range of reasonable pressures (e.g., 10 - 30 psi), repetitive blasts or combined primary and secondary insults (e.g., blast followed by weight drop induced skull-concussion). Others have shown that repetitive low level blasts, head concussions, or blunt force trauma to the eyes alone can lead to severe retinal degeneration in mice and rats (Blanch, 2012, 2014; Tzekov, 2014; Choi, 2015).

Also, while behavioral impairments in visual acuity tracking reflex (i.e., optokinetics) have been looked at (Hines-Beard, 2012; Bricker-Anthony, 2014b), no one has attempted to translate the retinal injuries into actual loss of performance on vision dependent psychomotor tasks. Indeed, for blasted rats, we saw a 30% decrease in retinal signaling with a 2 and 3-fold more neuronal cell damage in their retinas and brain optic tracts, respectively; however, most rats still performed quite well on the visual discrimination task. Thus, mostly trends in vision related behavioral deficits have been observed so far. This test does have the limitation that it is impossible to be certain that the animal doesn't try to work around the test, such as compensating with other senses heightened by loss of sight (e.g., hearing the cue light relay switches go off). There is also a concern that the rat's capacity for memory and learning may play a larger part than considered in the test's outcomes. Improvements of visual behavioral testing by us in the future will include using a battery of functional tasks, such as novel object recognition, cued maze navigation, and spatial place preference (Crawley, 2007). We will also add visual acuity measurements by optokinetics to our repertoire of tests, since it is purely based on reflexive response to a rotating bar pattern that requires no training of the animals (Douglas, 2005). Recent pilot experiments by us with the device have demonstrated a 50% loss in visual acuity occurs in single blasted rats within 7 days post-exposure (e.g., 0.25 vs. 0.10 cycles/degree). Additionally, we will do ERGs with light pattern stimulation (pERG), which looks at signals from retinal cells involved specifically in visual acuity processing (i.e., ganglion cells) or visually evoked potentials (VEPs) after similar stimulus, as recorded as electroencephalograms (EEGs) from the brain's occipital cortex (Perlman, 2009).

Our histopathology assessments looked at simple changes in neuronal cell morphology as a sign of degeneration only at the chronic time point of 14 days post-exposure and we do not know what more complex and earlier cellular events happened (e.g., apoptotic proteins), which also is helpful for shaping the therapeutic "window". Improvements of the histopathology by us in the future will be to look post-blast at acute time points (e.g., 6 and 24 hours) and semi-chronic time points (e.g. 3 and 7 days); where some specialized immunohistochemistry based-stains for more acute damage would be TUNEL (DNA damage) and Iba-1 or CD68 (immune cell infiltration) (Naskar, 2002; Nakazawa, 2006; Bailes, 2010). It would also be interesting to look at chronic time points far beyond 14 days post-blast (e.g., 21 and 28 days), since ERG exams and visual discrimination testing indicated that some recovery of visual function was occurring by then. This, however, may be a transient rebound phase with the injury state gradually worsening thereafter. Progressively slow neurodegenerative diseases, originating from a blast induced insult, are known for the brain, such as chronic traumatic encephalopathy (CTE) (Goldstein, 2012). Histopathology of the retina and brain visual processing centers at far time points post-blast could also look for "classic" biomarker proteins of chronic neuro-degeneration, such as p-tau, β-amyloid, and GFAP (Hoshino, 1998; Cao, 2001; Liberto, 2004; Griciuc, 2011).

Our project is currently limited to neuro-physiological, behavioral, and pathological outcome measures assessed at up to 14 days post-injury and does not allow us to adequately address the biochemical alterations behind any negative changes observed, which could lead to new targets for drug candidate considerations. Characterization of these changes will require future Western blot, ELISA, or immunoassay array (e.g., Luminex assay) evaluations of specific proteins in fresh retina and brain tissues collected from animals over a finely divided and extended timeframe post-injury to capture both acute and chronic biochemical effects (e.g., 6 hours, and 1, 3, 7, 14, and 28 days). Proteins examined could be selected from those well recognized as biomarkers of neuroinflammation mediated apoptosis (e.g., COX-2, bFGF, IL-1β, MCP-1, caspase-3, and TNF-α) and retinal signal transduction (e.g., rhodopsin, Gt-α, and cGMP-PDE) (Cao, 2001; Nakazawa, 2006; Rapoport, 2008; Bailes, 2010; Haung, 2012). Plasma collected from blasted rats could also be screened for these proteins to see if there is a correlation with retina and brain levels, as a non-invasive diagnostic tool for judging the presence of neuronal injuries.

Despite shortcomings in some of the outcome measures, our studies provided us an excellent blast wave induced injury model for testing the efficacy of experimental drug therapies to alleviate the neuronal cell damage to the retina and brain visual centers. Only two studies in the literature have demonstrated that drug interventions, using nicotinamide phosphoribosyl transferase and β-adrenergic receptor agonists (i.e., proprietary compounds, as structurally based on adenine nucleotides and isoproterenol, respectively), can prevent retina inflammation and cell degeneration in rats exposed to blast waves (Jiang, 2013; Ducta, 2014). These investigators treated the blasted eyes using topical application of the drugs to the cornea or their intraperitoneal injection, which are slow and inefficient absorption routes that eventually cover the retina and less likely the brain visual centers, due to blood-brain barrier crossing issues. In our study, we gave blasted rats intravenous injections of one of four experimental drugs, i.e., lipoxin A4, protectin DX, resolvin D1, and resolvin E1, that are well known to be very potent pro-resolving lipid mediators of neuro-inflammation in both the brain and the retina after mechanical insults (Serhan, 2008; Bazan, 2010; Serhan, 2010). We had considered intravenous injection to be one of of the most medically practical ways, in the field or clinic, to get these drugs rapidly on board to injury sites found both within the retina and brain. To fully saturate the injury sites we even injected the drugs immediately post-blast at a recommended effective dosage (25 μg/kg) and then followed by repetitive booster injections out to 14 days. Independent outcome measures used to carefully assess visual system function and neuronal cell health in both the brain and the retina (i.e., ERG, visual discrimination testing, and histopathology), however, failed to indicate that these drugs were producing a robust effect at preventing neuronal cell degeneration. Interestingly, all outcome measures showed widely different drug efficacy orders, with at best hints of a highest all around efficacy (i.e., lipoxin A4) and very scattered or modest improvements in blast injury recoveries. While our final group sizes for each drug are rather low (n = 11 - 12) and could be greatly powered up, this lack of even a modest demonstration of blast-intervention efficacy for all four drugs has left us greatly puzzled and looking for experimental design flaws.

It could be that the diffusive axonal shearing nature of the blast injuries that we are dealing with is not amendable to the pharmacological activity of this class of drugs, and thus doesn't really require the detrimental activities of infiltrating immune cells to trigger cellular apoptosis of the damaged neurons. How to deal with this scenario is uncertain, other than pursuing new types of drug targets, such as structural proteins (e.g., amyloid-β and tau) involved in maintaining axonal integrity (Hoshino, 1998; Goldstein, 2012). Failure of our therapeutic approach, however, is more likely due to ineffective delivery of these drugs to the neuronal injury sites from systemic dilution into non-specific tissue compartments. Stability could be another factor, since these compounds are sensitive to oxidative processes and thus have a very short in vivo half-life. Endogenously, they are also maintained at extremely low concentrations in the tissues. This is why we did not attempt to do pharmokinetic experiments to measure their blood or tissue levels after injection (e.g., by LC/MS). It could also be a problem of uptake into the retina and brain visual centers, in light of the non-lipophillic (polar) nature of these hydroxylated compounds. While the choroid and retinal pigmented epithelium readily take up neuroprotectins and resolvins from the blood and secrete them to the retina photoreceptor cells (Connor, 2007; Bazan, 2010), the efficiency at which they are able to cross the blood-brain barrier is uncertain (Marcheselli, 2003). Disruption and increased permeability of the blood-brain barrier seen following blast exposure may not be widespread or long enough to allow a saturating passage of polar drugs to occur (Readnower, 2010; Svetlov; 2010; Garman, 2011). Indeed, therapeutic testing of neuroprotectins in rodent models of stroke has relied on direct introduction into the brain by intracerebroventricular (i.c.v.) infusion to produce favorable outcomes (Marcheselli, 2003).

It is not the case that we mistakingly selected drugs without great therapeutic potential. Lipoxin A4, has recently proved highly efficacious in reducing lesion volumes, edema, cytokines, and apoptotic proteins in the brains of mice given a controlled cortical impact (CCI) injury, but again its introduction had to be done directly into the brain by i.c.v. injection (Luo, 2013). Likewise, mice subjected to brain damage by lateral fluid percussion injury (FLPI) showed marked improvements in neuro-behavioral function (e.g., rotarod) and suppressed neuro-inflammation responses (e.g., microglia activation), when intraperitoneal injected with resolvin D1 and resolvin E1 (Harrison, 2015); however, to see this effect, both prophylactic and post-injury administration of the drugs had to be done (i.e., 3 days before and after, at 5 μg/kg). It also must be considered that the brain damage used in the two studies above are more shallow focal point injuries and not of the deep diffusive nature that our blast wave model produces; and thus, they are easier to

effectively target and heal. Finally, we will try experiments using specialized delivery platforms to ferry our drugs across the blood-brain barrier and saturate many neuronal injury sites, after intravenous injection post-blast; such as by packaging them in liposomes or dendrimer based nanoparticles, which has worked well with many other drugs for treating neurodegenerative diseases of the brain and retina (Navath, 2010; Iezzi, 2012; Kannan, 2012).

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

Peer-Reviewed Scientific Journals:

* On 29 June 2015 submitted a manuscript for the first part of the study, which was general characterization of the blast-induced visual system injuries, to the Journal of Neurotrauma. The title was "Effect of primary blast over pressure on retina and optic tracts in rats", and authors were James DeMar, MAJ Keith Sharrow, Miya Hill, Andrea Edwards, Joshua Berman, COL Thomas Oliver, and Joseph Long. On 05 August 2015, we received a letter of full rejection, mainly due to minimal outcome measures, subtle neuronal injury effects, and lack of mechanistic elucidation. The paper is currently being revised with consideration to the reviewer comments and then will be resubmitted to the alternative journals of Investigative Ophthalmology and Visual Sciences (IOVS) and/or Frontiers in Neurology.

Invited Articles:

The overall project and its goals for advancing Military Medicine were detailed in an article found in the Geneva Foundation's annual report newsletter, released in September, 2013. A copy of the article is attached to this report.

Conference Abstracts and Presentations:

1) * Abstract and poster presentation at the Military Health Systems Research Symposium (MHSRS), held in Ft. Lauderdale, FL on 12 -15 August, 2013. The title was "Evaluation of novel polyunsaturated fatty acid derived mediators of inflammation to ameliorate primary blast wave induced injuries to the visual system of rats" and the authors were James DeMar, Miya Hill, Robert Gharavi, Joseph Andrist, Andrea Edwards, and Joseph Long.

2) * Platform presentation to the Geneva Foundation's Scientific Advisory Board during their site visit to the WRAIR on 11 July, 2013. The title was "Evaluation of novel polyunsaturated fatty acid derived lipid mediators of inflammation to ameliorate the deleterious effects of blast over pressure on eye and brain visual processing centers in rats" and the authors were James DeMar, Miya Hill, Stephen VanAlbert, and Joseph Long.

3) * Abstract and poster presentation at the National Capital Area Traumatic Brain Injury Research Symposium, held at the NIH in Bethesda, MD on 03 - 04 March 2014. The title was "Exposure to primary blast waves causes traumatic injury to the visual system in rats" and the authors were James DeMar, Stephen VanAlbert, Miya Hill, Robert Gharavi, Joseph Andrist, Andrea Edwards, Cory Riccio, and Joseph Long. As this is a final summation of the first phase of the study, which was characterization of the blast-induced visual system injuries, a copy of the submitted / accepted abstract and poster are attached to this report.

4) * Abstract and poster presentation at the 32nd National Neurotrauma Symposium , held in San Francisco, CA on 29 June - 02 July 2014. The title was "Characterization of a blast-induced brain

and eye injury model in rats" and the authors were MAJ Keith Sharrow, James DeMar, Miya Hill, Andrea Edwards, Joseph Long, and COL Thomas Oliver.

5) Abstract and poster presentation at the National Capital Area TBI Research Symposium, held in Bethesda, MD on 09 - 10 March 2015. The title was "Evaluation of novel polyunsaturated fatty acid derived mediators of inflammation to ameliorate primary blast wave induced injuries to the visual system of rats" and the authors were James DeMar, Miya Hill, Robert Gharavi, Joseph Andrist, Andrea Edwards, Donna Wilder, Meghan Mccuistion, and Joseph Long.

6) Abstract and poster presentation at the 33rd National Neurotrauma Symposium, held in Santa Fe, NM on 28 June - 01 July 2015. The title was "Evaluation of novel polyunsaturated fatty acid derived mediators of inflammation to ameliorate primary blast wave induced injuries to the visual system of rats" and the authors were James DeMar, Miya Hill, Robert Gharavi, Joseph Andrist, Andrea Edwards, Donna Wilder, John Rosenberger, Meghan Mccuistion, and Joseph Long.

7) Abstract and poster presentation at the Military Health Systems Research Symposium (MHSRS), held in Ft. Lauderdale, FL on 17 -20 August, 2015. The title was "Evaluation of novel polyunsaturated fatty acid derived mediators of inflammation to ameliorate primary blast wave induced injuries to the visual system of rats" and the authors were James DeMar, Miya Hill, Robert Gharavi, Joseph Andrist, Andrea Edwards, Donna Wilder, John Rosenberger, Meghan Mccuistion, and Joseph Long. As this is a final summation of the second phase of the study, which was testing four novel anti-inflammation drugs to ameliorate the blast induced visual system injuries, a copy of the submitted / accepted abstract and poster are attached to this report.

INVENTIONS, PATENTS AND LICENSES

There is nothing to report for this section.

REPORTABLE OUTCOMES

1) Developed an animal model, using adult male rats, for blast wave induced injuries to the visual system, which includes the retina and brain centers (e.g., optic tracts). Unlike other similar ocular trauma rodent-models in the literature, this is one of only three others to utilize high fidelity simulated blast over pressure waves (Friedlander waveform), as generated by a compressed air driven shock tube, to produce the injury. The outcome measures that we used were similar to those by others, but with more refined time points and closer interconnections.

2) Developed an animal model, using adult male rats, for testing the efficacy of experimental drugs against blast wave induced injuries to the visual system, including the retina and brain components. In this model, we administered the drugs via an intravenous route, which is one of the most medically practical ways, in the field or clinic, to get therapeutics rapidly on board to injury sites found within the retina and brain. No other studies have chosen an intravenous route for experimental drug delivery, where only two have attempted to treat air blast induced injuries to the eye using topical application to the cornea or systemically by intraperitoneal injection. Both of these are slow and inefficient absorption routes for the drugs to reach the retina as well as brain, mostly being sequestered in the intraocular space (i.e., corneal) or liver (i.e., intraperitoneal). Also, we are the first to test metabolites of omega polyunsaturated fatty acids as therapeutic drugs against inflammation involved in blast-induced injuries to the visual system. The two other studies examined drugs that were agonists towards the β-adrenergic receptor and nicotinamide phosphoribosyl transferase, respectively.

OTHER ACHIEVEMENTS

1) Nominated for the Geneva Foundation's 2014 Researcher of the Year award, which is presented to a researcher who exemplifies their mission of advancing innovative medical research within the U.S. military, for the benefit of U.S. service members and veterans, their families, and the global community. I was within the top twelve nominees, as chosen by Geneva's Scientific Advisory Board, but was not selected as one of the six finalists for the award.

2) Using preliminary data from the project's first phase, submitted a grant application to a DMRDP FY13 CRM-ARATDA sponsored program (on 9 September, 2013) as co-PI under Dr. Long, proposing treatment of blast induced ocular injuries with dietary supplementation of omega-3 polyunsaturated fatty acid. However, after grant board review our application was rejected for funding, as mainly based on issues regarding potential of treatment success.

3) Using preliminary data from the project's first and second phases, submitted a grant application to a CDMRP - USAMRMC sponsored vision research translational medicine award program (on 15 December, 2013) as a PI under Dr. Long, proposing to use nanoparticle delivery (intravenous) of metabolites of omega-3 polyunsaturated fatty acids (i.e., neuroprotectins and resolvins) for treatment of blast induced neurotrauma to the visual systems in rats. However, after grant board review our application was rejected for funding, as mainly based on issues of lack of clinical readiness and pharmokinetic or toxicity studies.

4) Using preliminary data from the project's first phase, submitted a grant application to a CDMRP - USAMRMC sponsored vision research hypothesis development award program (on 15 December, 2013) as a PI under Dr. Long, entitled "Elucidation of Inflammation Processes Exacerbating Neuronal Cell Damage to the Retina and Brain Visual Centers as a Quest for Therapeutic Drug Targets in a Rat Model of Blast Overpressure Wave Exposure". This study will involve advanced characterization of blast wave injuries to rat retina and brains, using advanced techniques of ERG, visual acuity testing (optokinetics), MRI and f-MRI (immune-cell tracking), histopathology, and cytokine array assays. We were successfully selected for $250K of funding over 2 years (FY14 - FY16) towards this project and assigned the award number W81XWH-14-2-0178, with the official start date on 30 September 2014.

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APPENDICES

Supplementary items that are attached to this report are copies of the 2013 Geneva Foundation's annual report newsletter; abstracts and accompanying posters that were presented at the 2015 National Capital Area Traumatic Brain Injury Research Symposium and the 2015 Military Health Sciences Research Symposium; and supplemental figures showing the number of animals completed for each outcome measure in the blast injury characterization and drug treatment studies; chemical structures of the four experimental drugs - lipoxin A4, protectin DX, resolvin D1, and resolvin E1 we tested; and the previously reported biochemical action of these experimental drugs for promoting wound healing.

Figure A: Tallies of animals completed for the study at each outcome measure

Blast injury characterizations:

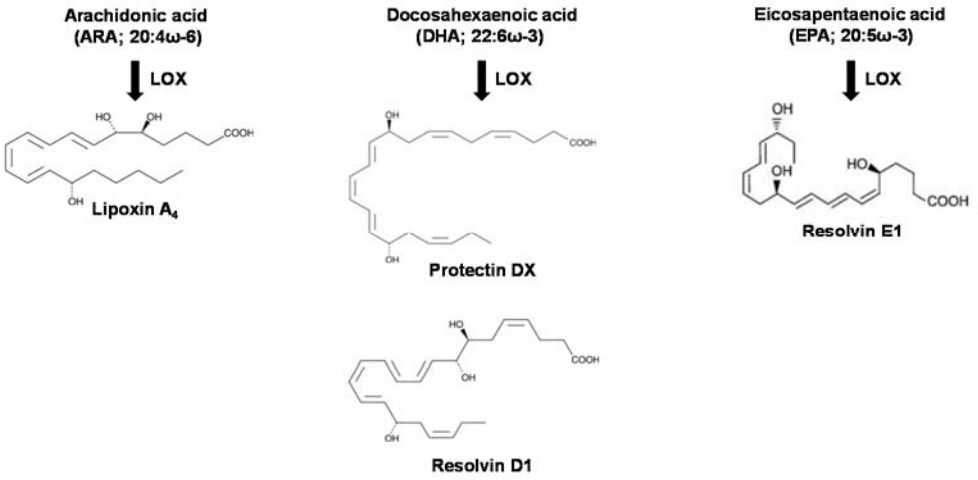
Treatments	ERG	Visual Discrimination	Retina and Brain Optic Tract Histopathology
Sham	**14**	**11**	**14**
Blasted	**15**	**10**	**15**

Experimental drug treatment following blast:

Treatments	ERG	Visual Discrimination	Retina and Brain Optic Tract Histopathology
Sham	**10**	**6 (naïve)**	**9**
Blasted cntrl	**22**	**11**	**22**
LXA4 + blast	**12**	**12**	**12**
PDX + blast	**11**	**11**	**11**
RVD1 + blast	**12**	**12**	**12**
RVE1 + blast	**12**	**12**	**12**

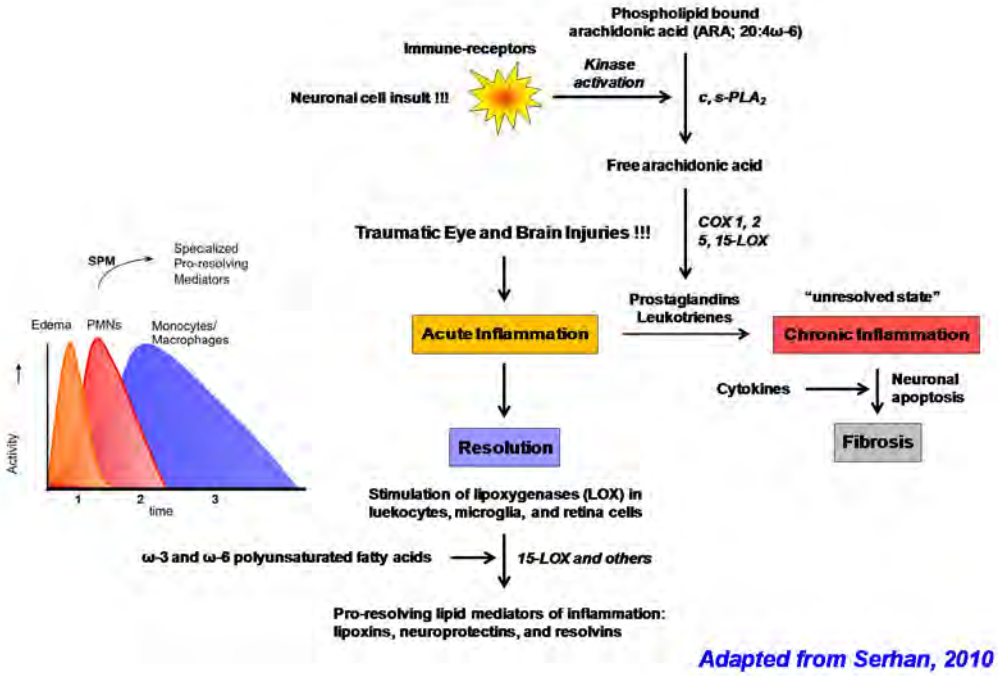
Supplemental Figure A: Final tallies of rats completed at each outcome measure for the blast injury characterization and experimental drug treatment following blast phases of the study. Outcome measures at up to 14 days post-blast were ERG, visual discrimination behavioral testing, and retina and brain optic tract histopathology. Experimental drug treatments were lipoxin A4, protectin DX, resolvin D1, and resolvin E1 (LXA4, PDX, RVD1, and RVE1, respectively). Amount of animals for each group ranged from 6 to 22 subjects. For the six shams done during the experimental drug treatment phase, naïve refers to rats that did not undergo any other procedures (e.g., ERGs or mock tail vein injections).

Figure B: Structures and endogenous pathway / source of experimental drugs



Supplemental Figure B: Chemical structures for the four experimental drugs used in this study are shown above, i.e., lipoxin A4, protectin DX, resolvin D1, and resolvin E1. All of these are stereo-specific hydroxylated derivatives of omega-3 and omega-6 polyunsaturated fatty acids. While we obtained their chemically synthesized forms from a commercial source (Cayman Chemicals Inc.), arachidonic acid (20:4ω-6) can be converted by endogenous lipoxygenase enzyme (LOX) activity to lipoxin A4; docosahexeanoic acid (22:6ω-3) to protectin DX and resolvin D1; and eicosapentaenoic acid (20:5ω-3) to resolvin E1. As shown below in supplemental figure 2, all of these molecules can target immune cell and turn off activities involved in inflammation processes that lead to chronic neuro-inflammation and thus promote healing of blast-induced injuries to the retina and brain visual centers.

Figure C: Pathways for progression and resolution of neuro-inflammation



Supplemental Figure C: Inflammation in the retina and brain post-blast injury can proceed in two directions. An initial acute inflammatory state of the nervous tissue can be stimulated to progress into a chronic state through rampant production of prostaglandins and luekotrienes from arachidonic acid (20:4ω-6). Arachidonic acid, which is esterified in phospholipids, is liberated by an immune factor-receptor mediated activation of phospholipase A2 (c or s-PLA2) through phosphorylation by receptor mediated kinases. Released arachidonic acid is converted by cyclooxygenases (COX 1 or 2) and lipoxygenases (5 and 15-LOX) to bioactive prostaglandins and leukotrienes, which are then secreted by these cells as secondary messengers to trigger global neuro-inflammation responses. These signaling molecules recruit cytokine releasing neutrophils to the site of injury, which leads to extensive destruction of perturbed neurons followed by necrosis and scarring (fiberosis) of the region. Alternatively, acute inflammation can enter a state of resolution. Prostaglandins can bind to receptors on leukocytes, microglia, and retina cells, which upregulate the gene expression of lipoxygenases (15-LOX) involved the production of pro-resolving mediators of inflammation from omega-3 and omega-6 polyunsaturated fatty acids. Arachidonic acid (20:4ω-6) can be converted by LOX activity to lipoxin A4; docosahexeanoic acid (22:6ω-3) to protectin DX and resolvin D1; and eicosapentaenoic acid (20:5ω-3) to resolvin E1. While these molecules have different cell receptor targets, in general their bioactivities are to turn off polymorphonuclear neutrophil actions and stimulate entry of monocytes / macrophages for wound clean up and healing to proceed. Above figure follows that of Serhan, 2010.

Abstract for the 2014 National Capital Area TBI Research Symposium:

Exposure to Primary Blast Waves Causes Traumatic Injury to the Visual System, in Rats.

James C. DeMar, Ph.D., Stephen A. VanAlbert, Miya I. Hill, Robert B. Gharavi, Joseph R. Andrist, Andrea A. Edwards, Cory A. Riccio, and Joseph B. Long

Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910

Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to non-penetrating traumatic injuries to the eyes or brain, likely caused by blast shock waves. In light of the difficult lifelong disability that permanent loss of vision represents, we propose there is a dire need to determine the degree of injury occurring specifically to the retina (e.g., photoreceptors) and brain visual centers (e.g., optic tracts), as result of exposure to blast waves. Using an adult rat model of blast wave exposure, we have now quantified the cellular and functional damage to the retina and brain, by electroretinography (ERG), visual discrimination behavioral testing, and histopathology. Blast wave injury was carried out by placing rats in a compressed air driven shock tube and exposing them once to a 20 psi (260 Hz) blast over pressure wave. Animals were then assessed at 1, 7, and 14 days post-injury. By 2 weeks out, blasted rats versus shams showed significantly decreased ERG waveform amplitudes, impaired ability to visually discern a cue light of variable intensity to earn food rewards, and severe neuronal cell degeneration within the retina and most brain visual processing centers (H&E and silver stains). Our research is an important contribution to providing the pathophysiological knowledge needed for developing therapies for blast related injuries and to advancing military medicine.

SUPPORT: This work is supported by a USAMRMC/ TATRC Vision Research Program grant award, #: W81XWH-12-2-0082.

Abstract for the 2015 Military Health Systems Research Symposium:

Evaluation of Novel Polyunsaturated Fatty Acid Derived Mediators of Inflammation to Ameliorate Primary Blast Wave Induced Injuries to the Visual System of Rats.

James DeMar, PhD[1], Miya Hill, BS[1], Robert Gharavi, BS[1], Joseph Andrist, BS[1], Andrea Edwards, BS[1], Donna Wilder, BS[1], John Rosenberger, BS[1], Meghan Mccuistion, BS[1], and Joseph Long, PhD[1].

[1]Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910.

Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss from non-penetrating traumatic injuries to the eyes or brain, caused by blast shock waves. In light of the difficult life-long disability that loss of vision presents, there is an urgent need for new drug therapies that can ameliorate the progression of neuronal degeneration in the eye (retina) and brain as the result of blast wave exposure. Our hypothesis is that novel metabolites of polyunsaturated fatty acids, known to be potent pro-resolving mediators of inflammation, i.e., lipoxins, neuroprotectins, and resolvins, will aid as drugs to promote healing of neurons critical to visual function after blast injury. In an adult rat model of blast wave exposure, we have utilized electroretinography (ERG), visual discrimination behavioral testing, and histopathology to thoroughly show that by 14 days post-blast, visual dysfunction occurs in association with underlying neuronal degeneration of the retinas and brain optic tracts. Blast injury was produced in anesthetized rats that were secured in a compressed air driven shock tube and then exposed to a single blast over pressure wave (20 psi peak, 8 msec duration). For neuroprotective drug evaluations, rats received one of four compounds, lipoxin A4, protectin DX, resolvin D1, and resolvin E1 (n = 12, each), which was intravenously administered immediately post-insult and then every other day out to 14 days. Likewise, shams and blasted controls were given saline vehicle injections. Retina and brain status were assessed using the procedures identified above. Surprisingly, our results suggest that these drugs afford slight if any protection against the neuronal degradation occurring within the blasted rat's visual system. Failure of this therapeutic approach is likely due to ineffective delivery of the drugs to neuronal injury sites as a result of systemic dilution, transient half-lives, and/or poor passage across the blood-brain / retinal barriers. These obstacles might be overcome using tissue targeted drug delivery platforms, e.g., nanoparticles, which if successful will provide an important therapeutic tool for blast injuries.

SUPPORT: This work is supported by a USAMRMC / TATRC Vision Research Program grant award, #: W81XWH-12-2-0082.

Developing Treatments for Blast-Related Vision Loss

"I can tell you, from my perspective, the signature weapon of this conflict is blast, and blast is a potentially devastating weapon which can burn, can result in amputation of limbs, that can result in loss of eyesight and hearing, that can damage brains and obviously, as we're all concerned, can lead, because of the context of the conflict for the combatant, to many post-traumatic stress results."

– *LTG Eric Schoomaker, Commander, USAMEDCOM, April 17, 2008*[1]

Blast injury from detonation of improvised explosive devices (IEDs) has emerged as the most frequent battlefield injury and greatest threat to warfighters in the current operations of Iraq and Afghanistan. Standard penetrating and blunt trauma to the body is the most common injury among survivors, and up to 10% of those afflicted have significant eye injuries[2]. Blast-related eye injuries often occur without any obvious outward signs of trauma, making them difficult to recognize, diagnose, and treat.

A leading cause of vision loss in the warfighter is the result of exposure to blast shock waves and the subsequent non-penetrating traumatic injuries to the eyes and brain visual processing centers[3]. A substantial portion of blast-related closed-eye injuries, up to 26%, involve tears, detachments, and hemorrhaging of the retinas.[4] Based on human clinical studies and recent animal studies, it is of high probability that exposure to even moderate blast waves can lead to neuronal cell death in the retina and brain visual processing centers that is severe enough to cause partial or full blindness.

Permanent loss of vision is a lifelong disability that has a profound impact on the warfighter's quality of life. In 2012, Dr. James DeMar, a Geneva researcher at the Walter Reed Army Institute of Research (WRAIR), began a research study to address the urgent need for new drug therapies to stop the progression of cell death in the retina and brain as a result of exposure to blast waves. This scenario is especially of concern when eye and brain blast injuries suffered by military personnel are not immediately attended to in the field, continuing the inflammation process and damage to the eye for an extended period of time. Dr. DeMar is specifically interested in studying novel drugs derived from omega-3 polyunsaturated fatty acids, which are known to be potent anti-inflammatory agents.[5]

The frequency of blast exposure and the resulting blast injuries from recent combat operations have allowed Geneva researchers to draw a more accurate clinical picture of the impact of blasts. The results of blast injury research have and will continue to be instrumental in improving the safety of our warfighters during combat, the quality of life for veterans, and even the well-being of civilians at job sites. This important research conducted by Geneva teams will continue to add to the growing base of knowledge in the treatment and prevention of injuries related to blast exposure.

1. *US Department of Defense, Blast Injury Research Program, https://blastinjuryresearch.amedd.army.mil/index.cfm?f =application.introduction (Apr. 29, 2011).*
2. *Centers for Disease Control and Prevention*
3. *Capó-Aponte JE, Urosevich TG, Temme LA, Tarbett AK, Navjit K, and Sanghera OD (2012). Visual dysfunctions and symptoms during the subacute stage of blast-Induced mild traumatic brain injury. Military Medicine, 177, 7:804.*
4. *Cockerham GC, Rice TA, Hewes EH, Cockerham KP, Lemke S, Wang G, Lin RC, Glynn-Milley C, and Zumhagen L. (2011). Closed-eye ocular injuries in the Iraq and Afghanistan wars. N Engl J Med. 364(22): 2172-2173.*
5. *Serhan CN. (2010). Novel lipid mediators and resolution mechanisms in acute inflammation: to resolve or not? Am. J. Pathol. 177(4): 1576-1591.*

Exposure to Primary Blast Waves Causes Traumatic Injury to the Visual System in Rats

[1,2]James C. DeMar, Ph.D., [1,2]Miya I. Hill, [1]Robert B. Gharavi, [1]Joseph R. Andrist, [1]Andrea A. Edwards, [1,2]Cory A. Riccio, [1,2]Stephen A. VanAlbert, and [1]Joseph B. Long

[1]*Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910;*
[2]*As Contracted Through The Geneva Foundation, Tacoma, WA 98402*

Background

Blast injury has emerged as arguably the greatest threat to War fighters in current theaters of operation, and is a leading cause of vision loss due to non-penetrating traumatic injuries to the eyes or brain, likely caused by blast shock waves. In light of the difficult lifelong disability that permanent loss of vision represents, we propose there is a dire need to determine the degree of injury occurring specifically to the retina (e.g., photoreceptors) and brain visual processing centers (e.g., optic tracts), as result of exposure to blast waves. Using an adult rat model of blast wave exposure, we have now quantified the cellular and functional damage to the retina and brain, by electroretinography (ERG), visual discrimination behavioral testing, and histopathology. Blast wave injury was carried out by placing rats in a compressed air driven shock tube and exposing them once to a 20 psi (260 Hz) blast over pressure wave. Animals were then assessed at 1, 7, and 14 days post-injury. By 2 weeks out, blasted rats versus shams showed significantly decreased ERG waveform amplitudes, impaired ability to visually discern a cue light of decreasing intensity to earn food rewards, and severe neuronal cell degeneration within the retina and most brain visual processing centers (H&E and silver stains). Our research is an important contribution to providing the pathophysiological knowledge for developing therapies for blast elated injuries and to advancing military medicine.

SUPPORT: USAMRMC / TATRC Vision Research Program grant, award #: W81XWH-12-2-0082.

Introduction

- In recent theaters of operation (OIF and OEF), 80% of the neurotrauma cases in U.S. soldiers resulted from attacks using improvised explosive devices (Warden, 2006).
- Blast injuries are a leading cause of loss of visual function in War fighters, due to trauma to the eyes and brain visual processing centers (Capó-Aponte, 2012; Cockerham, 2011).
- Of these afflicted patients, 43% display closed-eye injuries (Cockerham, 2011).
- Of the ocular injuries, 26% involve the retina, consistent with a blast wave displacement of fragile tissues (Cockerham, 2011).
- Despite the serious life-long disability loss of vision represents, relatively few animal studies have been done to characterize neurotrauma to the visual system resulting from blast wave exposure (Petras, 2007; Hines-Beard, 2012; Jiang, 2013; Mohan, 2013; and Zou, 2013).

References:
Capó-Aponte et al., 2012; Mil. Med. 177(7): 804-813.
Cockerham et al., 2011; N. Engl. J. Med. 364(22): 2172-2173.
Hines-Beard et al., 2012; Exp. Eye Res. 99: 63-70.
Jiang et al., 2013; J. Neuroinflammation. 10: 96-102.
Mohan et al., 2013; Invest. Ophthalmol. Vis. Sci. 54(5): 3440-3450.
Petras et al., 1997; Toxicology. 121(1): 41-49.
Warden, 2006; J. Head Trauma Rehabil. 21(5): 398-402.
Zou et al., 2013; J. Neuroinflammation. 10: 79-99.

Aim of Study

Rigorously, characterize in rats exposed to high fidelity simulated blast overpressure waves the cellular, neuronal signaling, behavioral pathology of injuries to the eyes - specifically retina - and brain visual processing centers, as by:

1) Electroretinography (ERG).
2) Visual discrimination (operant conditioning).
3) Histopathology (H&E and silver stains).

Materials and Methods

Simulation of Primary Blast Wave Injuries:

- Adult male Sprague Dawley rats (6 wk-old) are exposed under isoflurane to blast over pressure waves, in a right-side on orientation, using a compressed air driven shock tube.
- Single air blast of ~20 psi is applied to the rat, via rupture of a Mylar membrane of predetermined thickness.

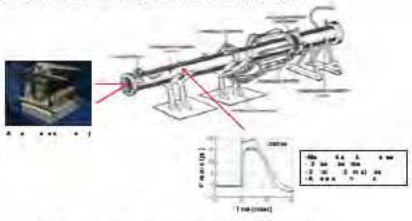


Figure 1. Diagrammatic view of the WRAIR shock tube.

Electroretinography (ERG):

- Rats are dark adapted for 5 h; and then kept under red lights.
- Under isoflurane, pupils are drug-dilated; and electrodes put on eyes (recording), cheeks (reference), and tail (ground).
- Eyes are flashed with light (0.1 - 25 cd.s/m^2; 5 msec); and evoked retina potentials are recorded (a- and b- waveforms).
- Tested at baseline (1 d prior) and 1, 7, and 14 d post-blast.

Figure 2. Rat mounted in an ERG instrument (Ocuscience, Inc.).

Visual Discrimination (Operant Conditioning):

- Rats are trained in operant conditioning boxes over 7 d to press a lever when a cue light shines to gain food rewards.
- Cue light is then varied in brightness (13 random levels) over next 2 d to challenge visual response, as a baseline prior to blast.
- Those having a ≥ 60% correct response are continued on.
- Retested at 2, 5, 7, 12, and 14 d after blast; and data is reported as total, correct, and incorrect lever responses.

Figure 3. Views of an operant conditioning box (Med Associates, Inc.).

Histopathology (H&E and Silver Stains):

- Rats are transcardial perfused with paraformaldehyde; and eyes and brains are removed and then post-fixed.
- Tissue samples are submitted (FD Neurotechnologies, Inc.) for processing into H&E (eyes) and silver (brains) stained slides.
- Examined under microscope for damage to retina and brain visual processing centers; where H&E stains for general cell morphology (pink to purple) and silver for axonal fiber tract degeneration (brown to black). Assigned relative damage scores on a scale of 1 - 6.

Results

Electroretinography (ERG):

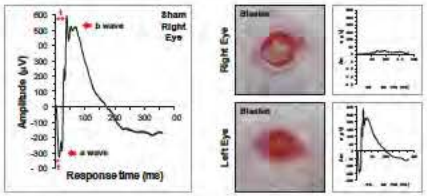


Figure 4. Electroretinogram (ERG) trace showing a- and b-wave responses (3 cd.s/m^2 flash), from retina photoreceptor and bipolar cell neurons, respectively; t = implicit time. Right and left eyes of a rat at 7 d post-blast, as shown along side their respective ERG traces.

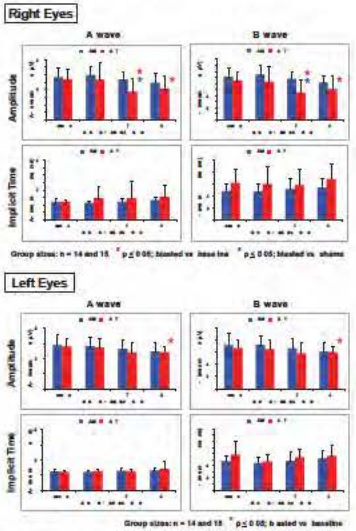


Figure 5. ERG amplitudes and implicit times for a- and b-wave signal responses (3 cd.s/m^2 flash) of sham and blasted rats (right and left eyes) at baseline and 1, 7, and 14 d after exposure. * $p \leq 0.05$, for blasted rats vs. their baseline or shams, as determined by t-test.

Visual Discrimination (Operant Conditioning):

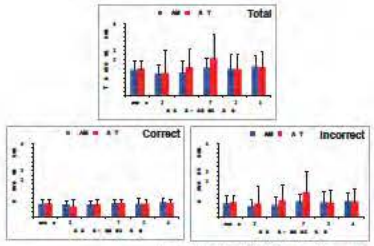


Figure 6. Visual discrimination test data for total, correct, and incorrect lever responses to a cue light in attempt to gain food rewards, as taken at baseline and 2, 5, 7, 12, and 14 d post-blast.

Histopathology (H&E and Silver Stains):

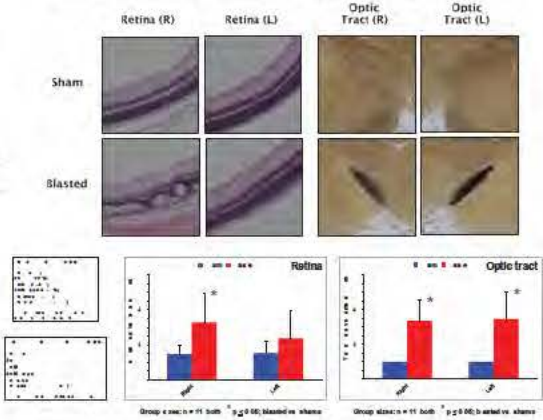


Figure 7. Histopathology of eyes (retina) and brains (optic tract) for sham and blasted rats; H&E and silver stains with relative damage scores, respectively. Magnifications are 4 - 10x. R = right; L = left.

Summary and Conclusions

- Blasted rats had significantly lower ERG exam a- and b-wave amplitudes at 7 and 14 d post-exposure, versus their baseline and sham values, which is a clear sign of retinal dysfunction.
- Visual discrimination testing showed a trend for the blasted rats to "guess" more for food rewards, over time similar to the ERG results.
- Histopathology showed cell damage to be present in the blasted rat retinas (degeneration) and brain optic tracts (axonal shearing).

Evaluation of Novel Polyunsaturated Fatty Acid Derived Mediators of Inflammation to Ameliorate Primary Blast Wave Induced Injuries to the Visual System of Rats

[1,2]James C. DeMar, Ph.D., [1,2]Miya I. Hill, [1]Robert B. Gharavi, [1]Joseph R. Andrist, [1]Andrea A. Edwards, [1,2]Donna M. Wilder, [1]John A. Rosenberger, [1]Meghan A. Mccuistion, and [1]Joseph B. Long

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THE GENEVA FOUNDATION

TATRC

Background

Blast injury has emerged as arguably the greatest threat to War fighters in current theaters of operation, and is a leading cause of vision loss due to closed injuries to the eyes or brain, likely from blast shock waves (Cockerham, 2011; Capó-Aponte, 2012; Lemke, 2013). In light of the difficult disability loss of vision represents, there is an urgent need for new therapies that can ameliorate neuronal degeneration in the eye (retina) and brain from blast wave exposure. Our hypothesis is that metabolites of omega-3 and -6 polyunsaturated fatty acids that are pro-resolving mediators of inflammation, i.e., lipoxins, neuroprotectins, and resolvins, will aid as drugs in healing neurons critical to visual function after blast injury. Previously, in a rat model of blast wave exposure, we showed by electroretinography (ERG), visual discrimination testing, and histopathology that visual dysfunction occurs along with neuronal degeneration of the retinas and brain optic tracts. In our current study, rats were exposed (right-side on) in a shock tube to a single blast over pressure wave (20 psi, 8 msec). For neuroprotective drug evaluations, rats were given lipoxin A4, protectin DX, resolvin D1, or resolvin E1 (n = 12, each) immediately after blast by i.v. injection, and then every other day for 14 days. Shams and blasted controls received saline injections. Retina and brain status were assessed using the outcomes identified above. Overall, our results suggest these drugs afford little if any protection against blast-induced neuronal degradation in the visual system. Failure is likely due to ineffective delivery to neuronal injury sites from systemic dilution, transient half-lives, and/or poor penetration of the blood brain / retinal barriers. These obstacles might be overcome by targeted drug delivery platforms, e.g., nanoparticles, which if successful will provide an important therapeutic tool for blast related injuries.

SUPPORT: USAMRMC / TATRC / VRP grant award #: W81XWH-12-2-0082.

Aim of Study

- While vision loss is a serious disability, only two animal studies have evaluated drugs (nicotinamide and β-adrenergic agonists) for blast induced neurotrauma to the eyes (Jiang, 2013; Dutca, 2014).
- Metabolites of omega-3 and -6 polyunsaturated fatty acids (hydroxylated) that are produced by immune cells, i.e., lipoxins, neuroprotectins, and resolvins can:

1) Promote wound healing by stopping progression from an acute to chronic phase of inflammation (Serhan, 2010, 2012).
2) Suppress neutrophil migration into tissues and their release of harmful eicosanoids and cytokines (Serhan, 2010; 2011).
3) Resolve physical injuries to retina and brain, when injected into rats and mice (Serhan, 2008, 2010; Luo, 2013; Harrison, 2015).

- To test the therapeutic efficacy of known metabolites of omega polyunsaturated fatty acids to ameliorate eye (retina) and brain injuries in rats after blast wave exposure. Outcome measures are ERG, visual discrimination, and retina / brain histopathology.
- Compounds representative of one lipoxin, one neuroprotectin, and two resolvins are tested in the blasted rats.
- Bottom line: Pro-resolving mediators of inflammation are introduced into blast-injured rats, by injection immediately after the insult; and thus, will readily promote neuronal cell healing.

References:

Capó-Aponte et al., 2012; Mil. Med. 177(7): 804-813.
Cockerham et al., 2011; N. Engl. J. Med. 364(22): 2172-2173.
Dutca et al., 2014; Invest. Ophthalmol. Vis. Sci. 55: 8330-8341.
Harrison et al., 2015; Brain Behav. Immun. 47: 131-140.
Jiang et al., 2013; J. Neuroinflammation. 10: 96-102.
Lemke et al., 2013; JAMA Opthalmol. 131(12): 1602-1609.
Luo et al., 2013; Brain Res. 1502: 1-10.
Serhan et al., 2008, 2010, 2011, & 2012; British J. Pharm. 153: 5200-5215, Am. J. Pathol. 177(4): 1576-1591, Curr. Top. Med. Chem. 11(6): 629-647, & Chem. Rev. 111(10): 5922-5943.

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- Single air blast of ~ 20 psi is applied to the rat, via rupture of a Mylar membrane.

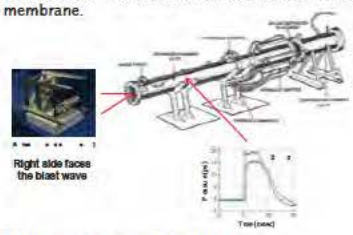


Figure 1. Diagrammatic view of the WRAIR shock tube.

Drug Injections:

- Experimental drugs, lipoxin A4 (LXA4), protectin DX (PDX), resolvin D1 (RVD1), and resolvin E1 (RVE1), are purchased from Cayman Chemicals. Purities are ≥ 99%, by QC analysis.
- Given immediately (< 5 min) following blast by i.v. injection (25 µg/kg) into lateral tail vein, and repeated every other day out to 14 days. Shams and blasted controls are given saline.

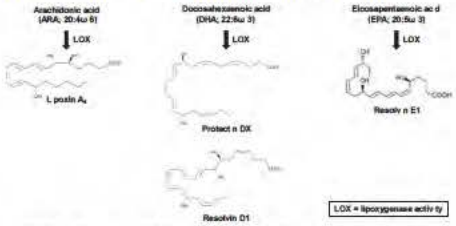


Figure 2. Structures and parent fatty acids of experimental drugs.

Electroretinography (ERG):

- Rats are dark adapted (≥ 5 h); and then kept under red lights.
- Under isoflurane, pupils are drug-dilated; and electrodes put on eyes (recording), cheeks (reference), and tail (ground).
- Eyes are flashed with light (3 cd.s/m²; 5 msec); and evoked retinal neuron potentials recorded (a- and b- waveforms).
- Tested at baseline (1 d prior) and 1, 7, and 14 d post-blast.

Visual Discrimination (Operant Conditioning):

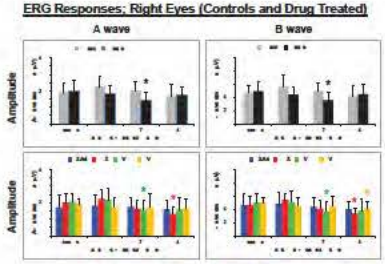
- Rats are trained in operant conditioning boxes over 7 d total to press a lever when a cue light shines to gain food rewards.
- Cue light is then varied in intensity (13 random levels) over next 2 d to challenge the rat, as a baseline prior to blast. Only those with ≥ 60% correct response rate continue testing.
- Retested at 2, 5, 7, 12, and 14 d after blast; and data is reported as total, correct, and incorrect lever responses.

Histopathology (H&E and Silver Stains):

- Rats are perfused with saline and then paraformaldehyde; and eyes and brains are removed and then post-fixed.
- Tissues are submitted (FD Neurotechnologies, Inc.) for processing into H&E (eyes) and silver (brains) stained slides.
- Sections are examined for neuronal cell damage to retinas and brain optic tracts, and scored on a rank scale of 1 - 6.

Results

Electroretinography (ERG); Right Eyes Only:



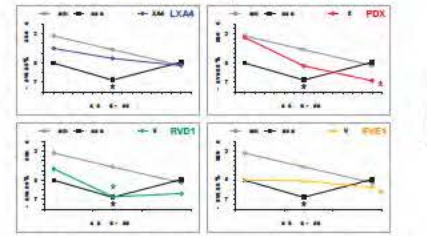
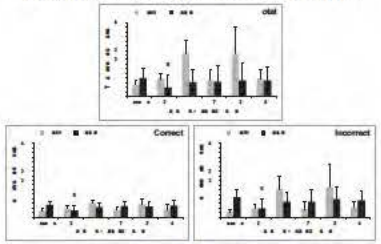
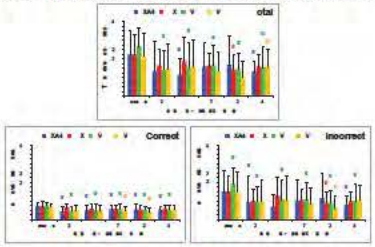


Figure 3. ERG amplitudes for a- and b-wave responses of sham, blasted control, and blasted drug-treated rats (right eyes only) at baseline and 1, 7, and 14 d after exposure. Mean ± SD. * p ≤ 0.05 vs. baseline.

Visual Discrimination (Operant Conditioning):





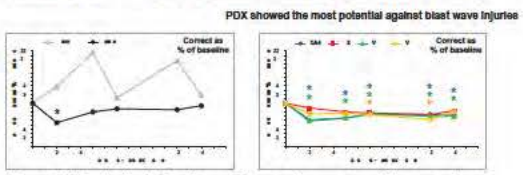


Figure 4. Visual discrimination test for total, correct, and incorrect lever responses of sham, blasted control, and blasted drug-treated rats, at baseline and 2, 5, 7, 12, and 14 d after exposure. Mean ± SD. * p ≤ 0.05 vs. baseline.

Histopathology; Right Retinas and Left Brains Only:

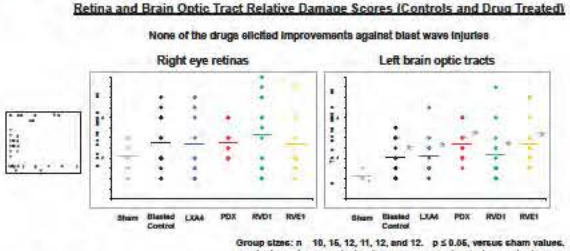


Figure 5. Histopathology damage scores for right eye retinas and left brain optic tracts of sham, blasted control, and blasted drug-treated rats at 14 d after exposure. Non-parametric data distributions (mean = bar). * p ≤ 0.05 vs. shams.

Summary and Conclusions

- Overall, the four drugs tested showed slight if any efficacy against blast wave induced neuronal injuries to the visual system of rats.
- ERG, visual discrimination, and histopathology outcomes yielded conflicting indications as to which drug has the most potential.
- Possible reasons for drug failure:

1) Ineffective delivery to the neuronal injury sites.
2) Each maybe better at targeting a different basis of the outcomes.
3) Nature of damage is not amenable to their activity following blast.